# Regulation of Zebrafish Metallothionein Gene Expression by Heavy Metal Ions

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**Abstract**

Metallothionein (MT) is a low molecular weight cysteine-rich intracellular metal binding protein for homeostasis of essential metals and detoxicfication of non-essential metal ion. Analysis of several fish MT genes has revealed conserved regulatory sequences, termed metal response elements (MREs), through which $Zn^{2+}$ activates the MT gene expression. Therefore zinc ion is commonly used as MT inducer; meanwhile the MT inductions by other heavy metal ions, such as arsenic and chromium, are not well studied. In this project, the relationships of $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Hg^{+}$, $As^{3+}$, $As^{5+}$, $Cr^{3+}$ and $Cr^{6+}$ with MT gene expression in zebrafish were studied.

To study the effect of different heavy metal ions on the MT gene expression, zebrafish (z) cell-lines, ZFL (liver cell) and SJD (caudal fin cell), were selected for our experiments. Zebrafish is a warm water species, it's biological and genetic studies were well characterized so as to provide a well developed database of zebrafish, making it become an ideal model in toxicological research.

The lethal effects of various heavy metal ions were firstly found in the two cell lines in order to give some preliminary data for further studies. When compare the toxicities of the metal ions in different valences, $As^{3+}$ was found more toxic than $As^{5+}$ while $Cr^{6+}$ is more toxic than $Cr^{3+}$.

The relationship between the heavy metal ions in interested with the MT gene expression was studied in the mRNA as well as the gene promoter level. Real time PCR technique was used to study the MT gene expression in mRNA level of the two cell lines after exposed into 10%, 25%, 50%, 75% and 100% $EC_{50}$ values of the heavy metal ions for 24 hours. Similar to the previous studies, $Zn^{2+}$and $Cd^{2+}$ were potent

MT inducers in the two cell lines. For other heavy metal ions, $As^{3+}$ and $As^{5+}$ are strong inducers in SJD cells only. $Cu^{2+}$ and $Hg^{2+}$ are moderate inducers while $Cr^{3+}$ and $Cr^{6+}$ are regarded as weak inducers in the two cell lines.

A 1.4kb long zMT-II promoter consists of 10 MREs and was amplified and cloned into pGL3-Basic vector for transient expression study following the administrations of heavy metal ions for 24 hours. $Zn^{2+}$ and $Cd^{2+}$ were found to be the most potent MT inducers in this study. $Ar^{3+}$ was a weak inducer in the two cell lines and $Hg^{+}$ can only cause significant induction in SJD cells, no significant induction was found in other heavy metal ion exposures.

Inductions of MTF-1 mRNA level by various heavy metal ions were also investigated. The result indicated that MTF-1 induction was induced by $Cd^{2+}$ in both cell-lines studied, however not all heavy metal ions regulate MT gene expression through this mechanism. $Zn^{2+}$ and $Cu^{2+}$, may activate the MT gene expression by facilitating nuclear translocation of MTF-1, phosphorylating MTF-1 and enhancing the MRE-MTF-1 binding.

This project investigated the inductions of MT mRNA levels of heavy metal ions that seldom studied and explored to the mechanism of MT gene expression by using SJD and ZFL cell lines as animal model. The long term goal of this project is to understand the relationship of MT gene expression with different heavy metal ions and to have a thorough understanding on the mechanism of MT gene expression controlled by various heavy metal ions.

## 摘要

金屬硫蛋白 (MT) 是一種低分子量並富含半胱氨的細胞內金屬黏合蛋白，作用於必需金屬的體內平衡和解毒非必需金屬。 當分析了幾種魚的 MT 基因後，有高度保存的調整基因序列展現了出來，它們名爲金屬反應元件 (MREs), 它們是透過鋅去活化 MT 基因表達。因此，鋅是一種很常用的 MT 誘導物， 但同時其他重金屬的誘導性，例如砷和鉻，則沒有深入研究過。這個科研項目將會探討鋅、鎘、銅、汞、三價砷、五價砷、三價鉻和六價鉻，和斑馬魚 MT 基因的關係。

爲了研究各種重金屬對 MT 基因表達的效應，斑馬魚細胞株 ZFL (斑馬魚肝細胞)和 SJD(斑馬魚鰭細胞)被選擇爲研究對象。斑馬魚是暖水種，而且它的生物遺傳特性已經有許多被認知，直接提供了一個良好的資料庫，令到斑馬馬成爲一種在毒理學上的理想模型。 這個項目首先找出各種重金屬離子對兩種細胞株的致命效應以爲深層研究提供一些初步資料。當比較那些擁有不用離子價的金屬離子的毒性，我們發現三價砷的毒性比五價砷高，而六價鉻的毒性比三價鉻。

各種重金屬離子和 MT 基因表達的關係，可在訊息核糖核甘酸(mRNA)和基因啓動子的水平上作出研究。爲了在研究 MT 在 mRNA 水平上的表達，兩種細胞株，均以各重金屬離子溶液的 10%, 25%, 50%, 75%和 100% 半數影響濃度處理了廿四小時，之後就用同步定量聚合酶鏈鎖反應去量度 MT mRNA 水平上的表達。而得出的結果和之前很多研究都一樣，鋅和鎘都是兩個最強的 MT 誘導者。而就其餘的重金屬離子而言，三價砷和五價砷在 SJD 細胞內是很強的 MT 誘導者，而銅和汞在 SJD 和 ZFL 細胞內是溫和的誘導者，三價鉻和六價鉻則在兩種細胞內均是弱的誘導者。

一段 1.4 kb 長的斑馬魚啓動子的序列，當中包含了十個 MREs，通過聚合酶

鏈鎖反應擴增克隆，並放進了 pGL3-Basic 載體裡，以用於報導基因檢驗。而啓動子的活躍度就在廿四小時未達至死量的重金屬屬離子處理後量度出來。同樣地，鋅和鎘在這項研究中都是表現得最強的誘導者。三價砷則在兩種細胞內都是表現弱的誘導者，汞就只有在 SJD 細胞內才可以表現出顯著的誘導。而其餘的重金屬離子則沒有表現顯著的誘導。

各金屬離子對金屬激活轉錄因子 1(MTF-1)在 mRNA 水平上的誘導性，都用同步定量聚合酶鏈鎖反應的技術去研究。結果發現，MTF-1 的誘導是一個可能的 MT 基因表達機制，主要同鎘離子有關，但並不是所有重金屬離子都會以這個機制去調節 MT 基因表達。如鋅和銅可能藉著以下三個機制去活化 MT 基因表達：幫助 MTF-1 易位到細胞核內，磷化 MTF-1 和提高 MRE 和 MRF-1 的黏合。

這個科研項目用 SJD 和 ZFL 細胞株作爲動物模型，探討了一些之前很少被研究的重金屬離子的 MT 誘導性並且對 MT 基因表達機制作多點了解。這個項目的長遠目標是要找出各種不同重金屬離子和 MT 基因表達的關係，從而對 MT 基因表達機制有一個更透徹的了解。

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## Abbreviations

| | |
|---|---|
| Ag | Silver |
| Al | Aluminium |
| AP1 | Activator protein-1 |
| As | Arsenic |
| Au | Gold |
| B | Boron |
| Ba | Barium |
| Be | Beryllium |
| Bi | Bismuth |
| Ca | Calcium |
| cDNA | Complementary cDNA |
| Cl | Chloride |
| Co | Cobalt |
| Cr | Chromium |
| Cs | Caesium |
| Cu | Copper |
| CYP1A1 | Cytochrome P-450-1A1 |
| dH2O | Double distilled water |
| EC50 | Effective concentration with 50% effect |
| EDTA | Ethylenediaminetetracetic acid |
| Fe | Iron |
| GSH | Gluthathionein |
| H2O2 | Hydrogen peroxide |
| Hg | Mercury |
| Li | Lithium |
| Luc | Luciferase |
| Mg | Magnesium |
| Mn | Manganese |
| Mo | Molybdenum |
| MRE | Metal responsive element |
| mRNA | Messenger RNA |
| MT | Metallothionein |
| MTF-1 | MRE-binding transcription factor-1 |
| Na | Sodium |

| | |
|---|---|
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| Ni | Nickel |
| PAH | Polycyclic aromatic hydrocarbons |
| Pb | Lead |
| PCR | Polymerase chain reaction |
| PKC | Protein kinase C |
| Pt | Platium |
| RNA | Ribonucleic acid |
| RT | Reverse transcription |
| Sb | Antimony |
| Se | Selenium |
| Sn | Tin |
| Sp1 | Specific protein-1 |
| Sr | Strontium |
| Te | Tellurium |
| Ti | Titanium |
| V | Vanadium |
| W | Tungsten |
| Zn | Zinc |
| ZnT-1 | Zin transport-1 |

**Abbreviations for Amino Acids**

| | | |
|---|---|---|
| A | Ala | Alanine |
| C | Cys | Cysteine |
| D | Asp | Aspartate |
| E | Glu | Glutamate |
| F | Phe | Phenylanine |
| G | Gly | Glycine |
| H | His | Histidine |
| I | Ile | Isoleucine |
| K | Lys | Lysine |
| L | Leu | Leucine |
| M | Met | Methionein |
| N | Asn | Asparagine |
| Q | Gln | Glutamine |
| R | Arg | Argnine |
| S | Ser | Serine |
| T | Thr | Threonine |
| V | Val | Valine |
| W | Trp | Tryptophan |
| Y | Tyr | Tyrosine |

# Chapter 1

# General Introduction

## 1.1 Metal Contaminations in the environment

The heavy metal elements make up a large part of the periodic table and include some of the most toxic agents, such as arsenic, mercury and cadmium, which are naturally distributed in rocks, soils, waters and air. However the metals were brought to the human through anthropogenic activities, in fact the progress of human civilization depends much on the use of metal. The expansion of the manufacturing industry during the Industrial Revolution brought about an increasing need for metals, a need which has continued to increase particularly in the $20^{th}$ century (Thornton and Abrahams, 1984). This increase of metal usage in the human activities has made human exposure to metals become unavoidable. Beyond this, the persistence nature of the metals makes them become more and more dispersed with high concentrations contaminating the environment.

Many human activities, such as mining operations, industrial process and landfill waste management with sludge and solid wastes, discharge heavy metals into the natural environment that can eventually enter to the water body, therefore many of the metal ions are known to exist in the sediments of water bodies, lakes and streams and

cause contamination to the fresh water and marine ecosystems (Shulkin and Kavun, 1995). The increases of heavy metal contamination in the aquatic environment post threats to human health. Examples are several thousands of elderly people in Japan suspected of experiencing *Itai-Itai* disease through cadmium poisoning in the 1950s (Uetani *et al.,* 2006) and several thousand of Japanese suffered from "Minamata disease" due to mercury poisoning (Mahaffey, 2005). These cases raised the awareness from the scientists and the behaviours of heavy metals in the ecosystems and their impact on living beings become our major concerns.

Many heavy metal ions may be accumulated in the human bodies and post harm on human health at chronic exposure levels, causing damages to internal organs such as brain, kidney and liver (Tandi *et al.,* 2004). Some metal ions like cadmium are possible carcinogen too (Bertin and Averback, 2006). These metal ions also pose harmful effects on other biological organisms in natural environment, including fish in the aquatic system which is the final sink of all contaminants on earth (Fraysse *et al.,* 2006). All in all, understanding the effects of metal ions on aquatic organisms, such as fish, would also provide much insight onto the possible impact of chronic exposures of metal ions on human.

## 1.2 Biology of Heavy Metal Ions

### 1.2.1 Essential and non-essential metal ions

Many metals are of concern because of their toxic properties, but some of the metal ions are also essential for survival and maintaining health of animal and human by playing special biological role(s). Calcium ion plays important role in muscle contraction, cell division, blood pressure and blood coagulation control, it is also a component of teeth and bone (Miggiano and Gagliardi, 2005).

Some heavy metal ions, like copper ion, are known as structural components for the metalloenzymes, acting as stabilizer to maintain the biological active structure of the enzymes (Zietz *et al.*, 2003). $Zn^{2+}$ also helps in stabilizing the structure of zinc-fingers in the transcriptional regulating protein and not only it could sever as Lewis acid for chemical reactions, it also facilitates hydroxyl nucleophile binding, forms tetrahedral structure at enzyme cleavage site for bond cleavage for catalysis (Delvin, 2002). Cupric ions also reduced by ascorbate acid to form reactive cuprous for oxygen to react with biomolecules in hydroxylation (Delvin, 2002).

Iron is a crucial component of haemoglobin in the red blood cells, it functions in transporting and storing of oxygen throughout the body, deficient in iron would lead

to anaemia (al-Othaimeen *et al.*, 1999). Although these metal ions are essential for life, they become toxic if the amount is in excessive in the human body; however the essential metal ions are tightly regulated in biological organisms with homeostatic control mechanisms.

Out of the 92 naturally occurring elements, approximately 30 metals and metalloids potentially toxic to human are: Be, B, Li, Al, Ti, V, Cr, Mn, Co, Ni, Cu, As, Se, Sr, Mo, Pd, Ag, Cd, Sn, Sb, Te, Cs, Ba, W, Pt, Au, Hg, Pb and Bi (Ortega, 2002). These heavy metal ions have no known biological role in human body and are not required in the living processes, they are regarded as the non-essential metals.

Non-essential metal ions become toxic when they replace the normal biological functions of essential metal ions. They can exert toxic effect to the human body even at low concentrations. However, Cr and Se are also regarded as essential elements as hormones and enzymes are associated with them. Chromium is found in insulin and may help the interaction of insulin with its binding proteins and receptors (Broadhurst and Domenico, 2006). Selenium is found in catalase for redox reaction and involved in the thyroid hormone metabolism (Kaprara and Krassas, 2006; Jaspers *et al.*, 2007). It functions through selenoproteins that contains selenocysteine at their

active site. The selenoproteins were identified and charaterized as the groups of glutathione peroxidase and thioredoxin reductase maintain cellular redox homeostasis and a group of iodothyronine deiodinases maintains thyroid hormone metabolism (McKenzie *et al.*, 2002; Jaspers *et al.*, 2007).

### 1.2.2 Toxicities and origins of heavy metal ions

The toxicity of heavy metal ions are determined by physiochemical of the metal ions and biological factors of the organisms. The physical and chemical properties of the metal ions, such as ion size, geometry, oxidation state, electro-negativity, bonding tendency, solubility and reactivity, are important factors that contribute to the toxic effects of metals and metalloids (Ariza, 1999). These factors determine the route that the metal ions enter into the body at the level of absorption. Metal intoxication can be lethal but very often causes sub-lethal effects including carcinogenicity, renal dysfunction, neuro-toxicity and hepatomegaly, depending on the organ or tissues being affected (Bertin and Averback, 2006; Brown *et al.,* 2006; Uetani *et al.,*2006; Bellinger *et al.,* 2007).

Many biological factors also affect the level of absorption of metal ions. Due to the hydrophobic nature of the cell membrane, metal ions cannot enter into the cell via

diffusion, but through the specific carriers, transporters and channels instead. For examples, $Cd^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Co^{2+}$ and $Ni^{2+}$ enter the cells through calcium dependent voltage ion channels or receptor activated channels, while chromate and arsenate enter as oxyanions through phosphate and sulphate carriers (Kiss and Osipenko, 1994; Dawson and Ballatori, 1995). Other methods include passive diffusion after forming organic complexes, transporting by neutral amino acid transport system, complexing to proteins that help in transporting the metal ions into the cell, and finally engulfed by phagocytosis (Jabado *et al.,* 2002). However, the uptake levels of each metal ion are not the same in different species and different tissues of the same species due to genetic differences in the absorption of metal ions.

Besides absorption, metal toxicity is also determined by metabolism, accumulation and excretion. Once the metal ions enter into the body, they would exert toxic effect to the organism through different mechanisms. The non-essential ions can replace the essential ions so as to affect normal cellular functions, for example the metal ions are able to bind with some chemical groups, such as amino, carboxyl, in cellular macromolecules (Kraemer *et al.,* 2005; McMeans *et al.,* 2007). The displacement of non-essential ions in these macromolecules would alter the structure of them and disrupt their functions. Another mechanism is to promote the

oxidative stress by depleting the small molecular weight thiols (such as glutathione), or binding to some enzymes which are important for antioxidant protection (Aravind and Prasad, 2005).

Some toxic effects are organ specific and the metal ion compartmentalized in the target organ(s), causing harm to these organs or tissues, for example $Cd^{2+}$ is known to be accumulated in kidney and cause renal dysfunction (Trzcinka-Ochocka *et al,* 2004). The tissue specificity also found at the sub-cellular level to exert toxic effects to the specific organelles, such as nucleus or mitochondria. It was found that the rodent cells showed higher $Cd^{2+}$ level in the nucleus than the other organelles after $Cd^{2+}$ absorption, in other words, the $Cd^{2+}$ accumulated in the nucleus of the rodent cells (Hechtenberg and Beyersmann, 1995; Lin *et. al.*, 1995).

Arsenic and chromium are two commonly found heavy metals in the aquatic environment and they are highly toxic to the human and aquatic organisms. These metals have different valances, where arsenic commonly exists as As (III) and As (V) and Cr (III) and Cr (VI) are the common forms of chromium ion. The released arsenic and chromium ions can enter into the water body due to their mobility, the contamination of these two heavy metal ions in the drinking water is gaining concern

in recent years. Both of the heavy metal ions can cause adverse effect to the human body and it is well known that these two heavy metal ions are carcinogens to human beings (Tseng *et al.,* 1968; Borgono *et al.,* 1977; Tseng, 1977; Cebrian *et al.,* 1983; Chen *et al.,* 1992; Smith *et al.,* 1992; Hopenhayn-Rich *et al.,* 1998; Rossman, 2003).

Arsenic is a heavy metal belonging to group 15 of the periodic table, it occurs naturally in many minerals with FeAsS being the most common (Hindmarsh *et al.*, 2002). The release of arsenic form natural source can be due to windblown dust from weathered continental crust, forest fires, volcanoes, sea spray, hot springs and geysers (Nordstrom *et al.*, 2000, Bhattacharya *et al.*, 2002). However, natural sources are only the minor sources of arsenic; the release of arsenic in the environment is mainly anthropogenic due to the commercial use of it: pesticides, wood preservatives, glass, alloys and electronics, etc (Greenwood *et al.*, 1989, Bhattacharya *et al.*, 2002).

Chromium belongs to group 6 in the periodic table, just like other heavy metal ions, human activity is the major source of its emission in the environment from waste incineration, fuel combustion, electroplating and leather tanning (Casadevall and Kortenkamp, 2002).

## 1.3 Monitoring Of Heavy Metal Contaminations In Aquatic Environment

Having reviewed the origin and toxicity of heavy metal ions, the situation of heavy metal contamination in the aquatic environment, monitoring of heavy metal ion contamination in the water body, especially in the drinking water or natural source of waters from ponds and streams for human consumption, are also important issues to the human health that are of our major concerns.

### 1.3.1 Monitoring in chemical approach

To monitor the heavy metal pollution in the aquatic environment, it can be done by accessing the amount of pollutant present in the water by chemical methods. Atomic Absorption Spectrum (AAS) and the Inductively Coupled Plasma (ICP) are used for the monitoring of heavy metal contamination, and the Gas Chromatography Mass Spectrometer (GCMS) and Ultra Violet- Visble (UV-Vis) spectrophotometer for the organic pollution assessment (Esteves *et al.,* 1999; Sallam *et al.,* 1999; Wurl and Obbard, 2005; Lin *et al.,* 2007).

Although chemical analysis is a simple and direct method of measurement of metal concentrations, when bioavailability is neglected, the amount of chemicals absorbed by the aquatic organisms is unknown and the effects of the chemicals on the

organisms are largely not known too. The chemical contents in the environment are always fluctuating, making accurate sampling a laboratorial work and difficult task.

The absorbed pollutants can pass to the higher trophic levels through the food chain with biomagnification effects, and it is also ignored by the chemical analysis of ecological samples like sediments. Besides absorption, the different mechanisms toward the pollutant (e.g. absorption, metabolism and excretion) can cause different bio-concentrations of the pollutant among the species; even they live in the same environment. Therefore analysis of chemical concentrations in the tissues of the organisms can show a better reflection on the level of absorption then measuring the content in the water body alone. The organisms that are chosen for chemical analysis are often known as biological indicators, such as fish, mussels and oysters, seaweeds in aquatic environment.

However, whether the absorbed metal ions can cause significant damage or sub-lethal toxic and chronic effects remain to be investigated. Hence, biomarkers are being developed for such diagnostic purposes to understand the possible toxic effects of chemicals posed on an organism.

### 1.3.2 Monitoring in biological approach: biomarkers

The exposure to pollutants would cause some measurable physiological changes, the measurement along individuals of their molecular, biochemical, or physiological parameters, are known as biomarkers (Jamil, 2001). Biomarker is an important concept in the bio-monitoring, as defined by the National Research Council, as "a xenobiotically induced variation in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample". The use of biomarkers can provide early warning signals of environmental deterioration and it is good for the study of long-term exposure at sub-lethal contamination of chemical agents, especially in the study of pollutant that is not persistent (Rand, 1995).

Accurate monitoring requires sensitive methods to evaluate dose-response relationships, especially at low concentration level exposures. The detection of subtle change of biomarker in gene level by Reverse Transcription Polymerase Chain Reaction (RT-PCR) is one of the methods developed with high sensitivity. It had been used to quantify CytochromeP-450-1A1 (CYPIAI) mRNA levels in human blood lymphocytes (Vanden Heuvel *et al.*, 1993), where CYPIAI is a biomarker of polycyclic aromatic hydrocarbons (PAH) such as bezopyrene and chlorinated PAH

such as polychlorinated dibenzodioxins. For an enzyme as CYP1A, enzyme activities could also be used as a biomarker. One of the most well used and standardized assays of P450 enzymes level is the measurement of the activity of an enzyme called EROD, 7-ethoxyresorufin o-deethylase (Klotz *et al.,* 1984). This enzyme converts ethoxyresorufin (7-ethyl-resorufin, 7-ER) to resorufin ***(deethylation)*** in the presence of NADPH and oxygen and the assay is well known as EROD assay. The amount of resorufin produced is measured fluorometrically in visible spectrophotometer at 572 nm after the addition of NADPH and the samples to be tested. The samples are often microsomal preparation from tissues because different cytochrome P450 isozymes could be found in the endoplasmic reticulum and microsomes of all animal cells.

However, measuring EROD is laborious and the enzyme activities are often inhibited by chemicals and thus measuring mRNA levels using RT-PCR is also a commonly used method to measure CYP1A levels with high sensitivity and reliability.

## 1.4 Metallothionein (MT)

### 1.4.1 Biological functions of MT and its regulation

For the study of heavy metal exposure, MT is a well characterized biomarker.

MT has been considered as a molecular marker of exposure to metal pollution in aquatic ecosystem (Chan, 1995). The first MT was found in equine renal cortex (Thirumoorthy *et al,*, 2007), since then other MTs or MT-like proteins have since been reported in many vertebrates including many species of fish (Olsson *et al.*, 1998; Amiard *et al.*, 2006).

MTs found in different vertebrate species are found to be highly conserved with a low molecular weight of 7 kDa, and each MT molecule has two subunits, the one is the more stable $\alpha$-unit, which is also called C-terminal and the other one is the more reactive $\beta$-unit, also named as N-terminal (Fig 1-1).

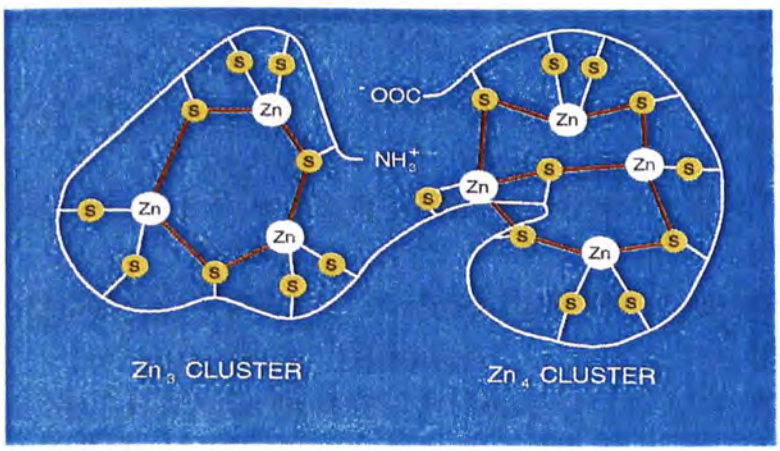



Fig. 1-1 The metallothionein molecule consist of 2 subunits, the N-terminal $\beta$-unit ($Zn_3$ cluster) and the C-terminal $\alpha$-unit ($Zn_4$ cluster).Four and three $Zn^{2+}$ are incorporated in $\beta$-and $\alpha$- unit respectively (adapted from Fischer and Davie, 1998).

The cysteine rich nature enables the MT protein to bind with metal ions intra-molecularly, each MT molecule can incorporate up to seven divalent metal ions or 12 monovalent $Cu^{+}$ ions per molecule (Nielson and Winge, 1985). Inside the cells, MTs function in essential metals homeostasis, such as $Zn^{2+}$ and $Cu^{2+}$ and the detoxification of non-essential elements, such as $Hg^{2+}$ and $Cd^{2+}$ (Fisher and Davie, 1998).

### 1.4.2 MT Isoforms

Different numbers of MT isoforms were reported from many different species. In mammals, there are 4 MT isoforms found (MT-I to MT-IV), and each MT isoform consists of a family of gene (Quaife *et al.* 1994). For example, there are 8 members under the MT-1 protein (MT-1A to MT-1L and MT-1X) and 2 MT-2 proteins (MT-2A and MT-2B) in the human body (Mididoddi *et al.,* 1996). The different isoforms showed differential expression in different tissues with MT-I and MT-II regulated co-ordinately in all tissues (Searle *et. al.,* 1984, Yagle *et al.,* 1985; Ghoshal *et al.*,1998), MT-III is brain specific (Palmiter *et al.*, 1992; Ghoshal *et al.*,1998) and MT-IV is expressed in the stratified squamous epithelium of skin and tongue (Quaife *el at.,* 1994; Ghoshal *et al.,*1998). Besides the tissue specificity, the regulations of different MT isoforms toward the heavy metal treatments are not the same (Klaassen

and Lehman-McKeeman, 1989).

In *Mytilus edilis*, MT-10 showed induction after the zinc, copper and cadmium treatments, but MT-20 only induced after the cadmium treatment (Vergani *et al.*, 2007). In other words, genes encoding distinct MT isoforms are differentially regulated by various heavy metal ions (Lemoine *et al.*, 2000).

Similar to other organisms, fish may have two MT isoforms, the MT-I and MT-II. These two MT isoforms have very similar amino acid sequences, the only point to classify them is at position 26, where MT-I is lysine (K) and MT-II is threonine (T) at least for the Cypriniformes (Fig. 1-2). In zebrafish, two MT isoforms were reported, MT II and MT A. The amino acid at position 26 is T in the two MTs, therefore it is believed that both of them are encoded for the MT II group, just like the MT-2A and MT-2B in the human body (Yan and Chan, 2004).

### 1.4.3 Mechanisms of MT gene regulation

MT expression is inducible by heavy metal ions. Although it has been demonstrated that MT is induced in fish by a variety of environmental chemicals – including paraquat and stress in some invertebrates, heavy metal ions such as $Cd^{2+}$,

$Zn^{2+}$, $Hg^{2+}$ and $As^{3+}$ are potent inducer of MT gene expression in different animal and plant species (Webb and Daniel, 1975; Cherian and Clarkson, 1976; Onosaka and Cherian , 1982; Carvan III *et. al.*, 2000).

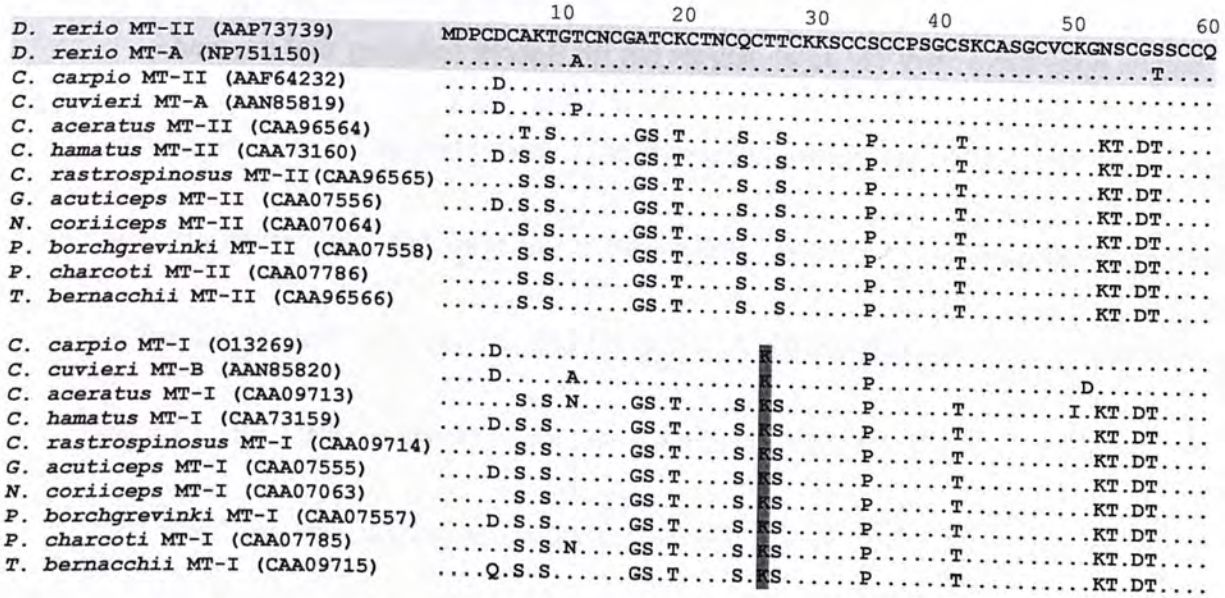



Fig. 1-2 Alignment of amino acid sequence of two MT isoforms in different fishes, the identical sequence is presented in dots (.). Threonine (T) is located at position 26 in MT-II isoform, which is different from the MT-I isoforms where lysine (K) is found instead (Yan and Chan, 2004).

In the current model of MT gene regulation, the metal responsive element (MRE) and metal transcription factor-1 (MTF-1) play important roles with MRE to act as a *cis*-acting element located in the MT gene promoter and MTF-1 as a *trans*-acting element and transcription factor to bind with $Zn^{2+}$. The binding of $Zn^{2+}$ leads to activation and translocation of MTF-1 from the cytoplasm to the nucleus acting and

targeting on the MRE in the promoter region of target genes; hence the transcription of MT gene is activated through these binding, mediation and gene activation activities (Lichtlen and Schaffer, 2001; Chen *et al.,* 2002, Laity and Andrews, 2007).

There are four proposed models for the regulation of MT gene expression related to $Zn^{2+}$ (Fig. 1-3). The first model is the allosteric model proposed by Heuchel *et al.*, 1995, suggesting that the increase in intracellular free $Zn^{2+}$ concentrations would activate the nuclear translocation and binding of MRE binding transcription factor-1 (MTF-1) with MREs in the MT promoter, and thus activate MT gene to express. It is believed that the activation is due to the binding of $Zn^{2+}$ to the zinc-finger of MTF-1 (Dalton *et al.*, 1997).

MTF-1 inhibitor model is the second model proposed for MT gene regulation by metal ions (Heuchel *et al.*, 1995). Under normal condition, MTF-1 is originally complexed with an inhibitor in this model. However, increase of $Zn^{2+}$ can free the MTF-1 from the inhibitor by disrupting the complex. The released MTF-1 is then freed to translocate into nucleus and activates the transcription of MT gene. The produced MT could bind with excess $Zn^{2+}$ and thus reduce the free $Zn^{2+}$ in the cytosol. Therefore the inhibitor-MTF-1 complex is allowed to reform and this can remove the activation of gene transcription and return the transcription level to normal level

(Heuchel *et al.*, 1995; Palmiter, 1995).

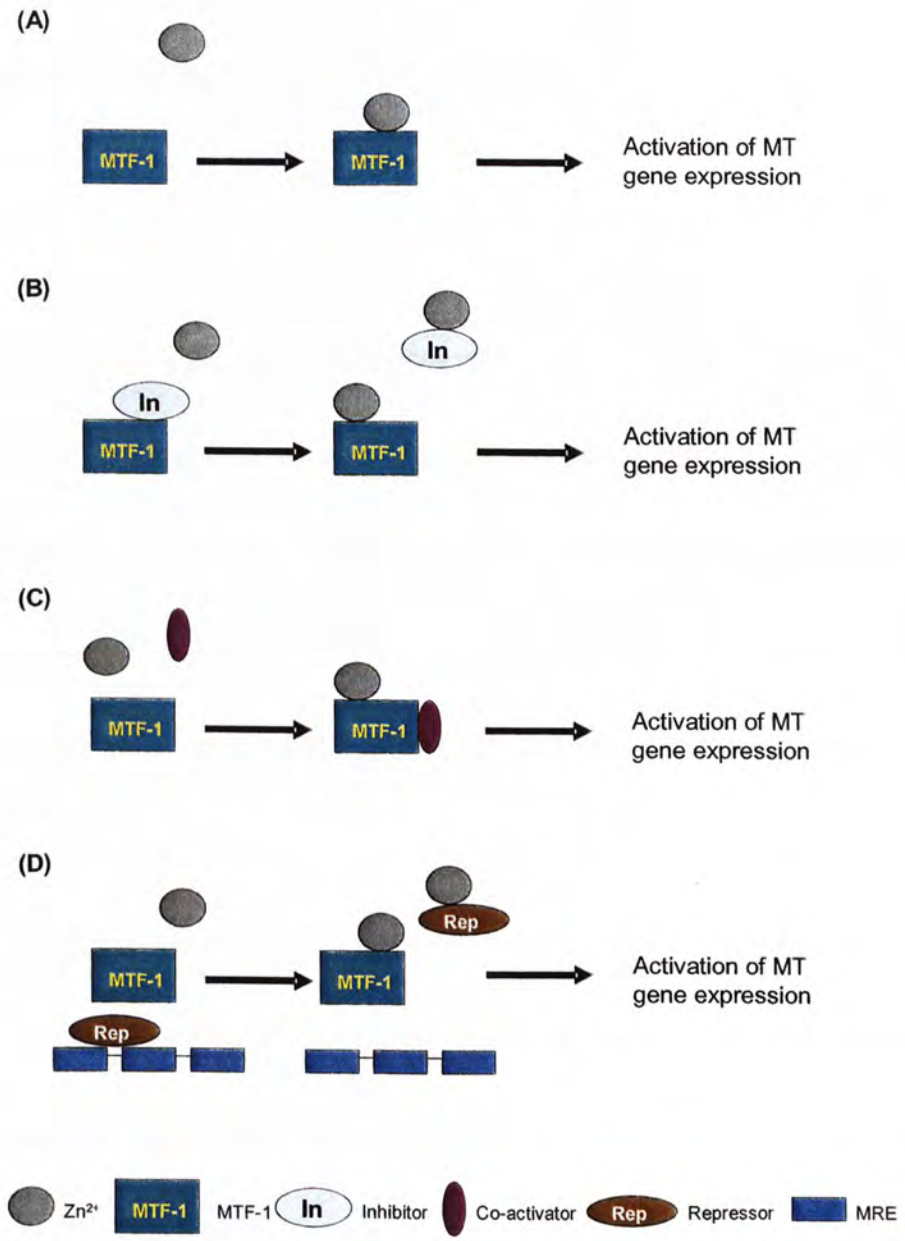



Fig 1-3 Four proposed models for $Zn^{2+}$ induced MT gene transcription. (A) allosteric regulation, (B) MTF-1 inhibitor model, (C) co-activator model and (D) DNA suppressor model (Modified from Heuchel *et al.*, 1995).

The third model is the co-activator model (Heuchel *et al.*, 1995). It suggested that with the increase of intracellular $Zn^{2+}$ concentration, MTF-1 would interact with an unidentified co-activator and then activated to bind with the MRE in the MT gene promoter.

The forth model is the DNA suppressor model (Fig. 1-3), which suggests that the MT promoter is originally bound with a repressor, making the MREs unavailable to MTF-1 (Heuchel *et al.*, 1995). With the increase of intracellular $Zn^{2+}$ concentration, the repressor would bind to the $Zn^{2+}$ and dissociate from the MT promoter, thus the MTF-1 is able to bind with the MRE for MT gene activation (Heuchel *et al.*, 1995).

These four models described above can only explain the up-regulation of MT gene expression caused by $Zn^{2+}$, but exposing to other heavy metal ions, such as $Cd^{2+}$ ion, can cause MT mRNA induction in zebrafish cell as well, despite $Cd^{2+}$ does not bind with MTF-1 (Dalton *et. al,* 2000). Perhaps MT can be induced through some indirect methods and the zinc-pool model and the phosphorylation model have been proposed for activation of MT gene by non-zinc ions.

### 1.4.3.1 Zinc pool hypothesis

The affinities of different heavy metal ions to the MT protein are not the same: $Hg^{2+}$> $Ag^{+}$> $Cu^{+}$> $Cd^{2+}$> $Zn^{2+}$ (Funk *et. al.*, 1984). Therefore, these different affinities allow the metal ions with higher affinity to enter into the MT protein and to displace $Zn^{2+}$; the MT gene expression is then preceded with these freed $Zn^{2+}$ which bind to MTF-1 for MT gene activation (Fig. 1-4).

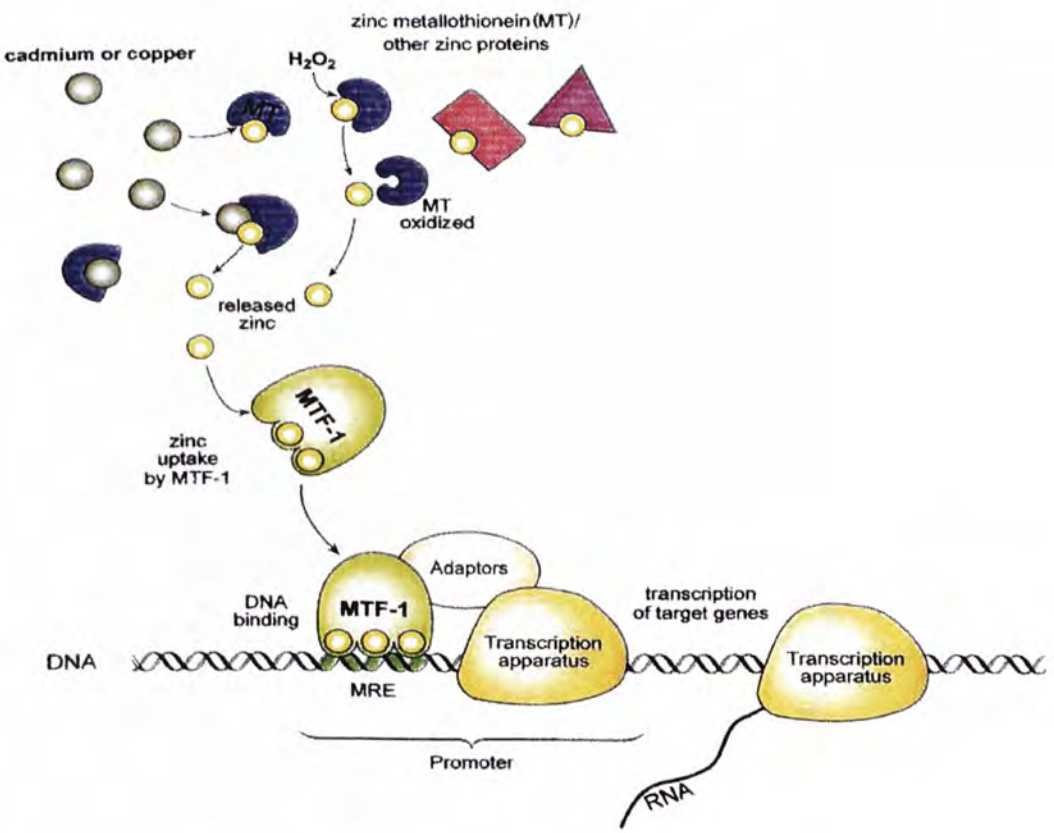



Fig. 1-4 Schematic diagram showing the zinc pool hypothesis on MT gene regulation by heavy metal ions. $Zn^{2+}$ incorporated in MT molecules is replaced by $Cd^{2+}$ and $Cu^{2+}$ due to the different infinity to MT molecule. (Adapted from http://mcb.asm.org/cgi/content/full/23/23/8471)

### 1.4.3.2 Protein kinase cascades

Besides zinc pool hypothesis, heavy metals ion other than $Zn^{2+}$ can activate MT gene transcription through some protein kinasing cascades. The extra metal ions present in the cell can activate several kinases like protein kinase C, and hence the MTF-1 is activated by these kinases through phosphorylations to give a stronger MT gene expression (LaRochelle *et al.*, 2001). The MTF-1 is phosphorylated at multiple sites at the serine and tyrosine residues. The protein is constitutively phosphorylated, and metal exposures enhance the levels of phosphorylation of MTF-1 (Fig. 1-5).

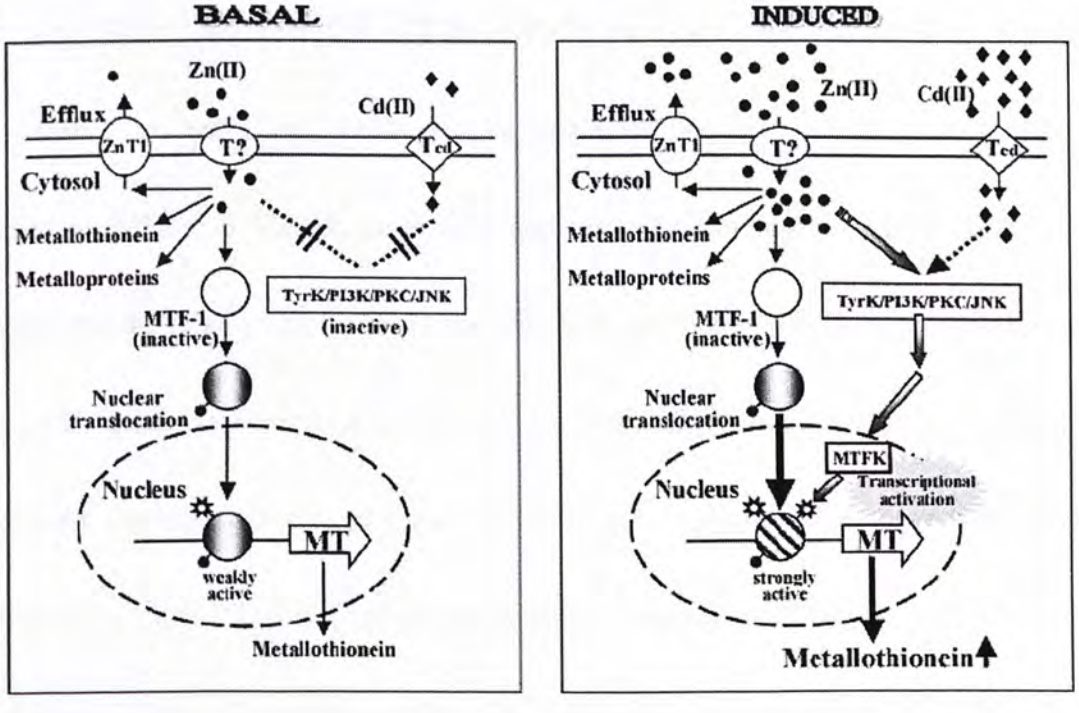



Fig. 1-5 MT expression controlled by metal-regulated protein kinase signal transduction cascade in basal and metal-induced conditions (LaRochelle *et al.*, 2001).

**1.5 Metal responsive element (MRE)**

MREs are the consensus short sequences located in the promoter region of all MT gene. They are present in multiple imperfect copies with a highly conserved core sequences 5'- TGCRCNC -3' (R = Purine, N = Any nucleotide). A wealth of information has been acquired on the structure and functional analysis of fish MT promoters, recent studies reported the promoter region of zebrafish and the relationship between the promoter region and the heavy metal ions were studied by the research groups.

An 835bp long zebrafish MT-II (zMT-II) promoter gene cloned from a local zebrafish (Yan and Chan, 2004) and the sequence was reported to have 4 MREs named as MREa to MREd, three AP1 and one SP1 binding sites (Fig. 1-6). To investigate the relationship between the zMT gene promoter with the heavy metal ions, the zMT gene was cloned into the pGL-3 Basic luciferase contruct to study the promoter activity after various metal ions exposure at sub-lethal dosages (0%, 25%, 50%, 75%, 100% 24h-$LC_{50}$ values) in transient transfection of HepG2 cell line (Yan and Chan, 2004). It was shown that zMT induction was caused by $Cd^{2+}$, $Cu^{2+}$, $Zn^{2+}$ and $Hg^{+}$, the inductions were dose-dependent (Fig. 1-7) and shown with time course effect (Fig. 1-8). Other chemical agents, $H_2O_2$, $Ni^{2+}$, $Pb^{2+}$ and $Co^{2+}$, did not cause

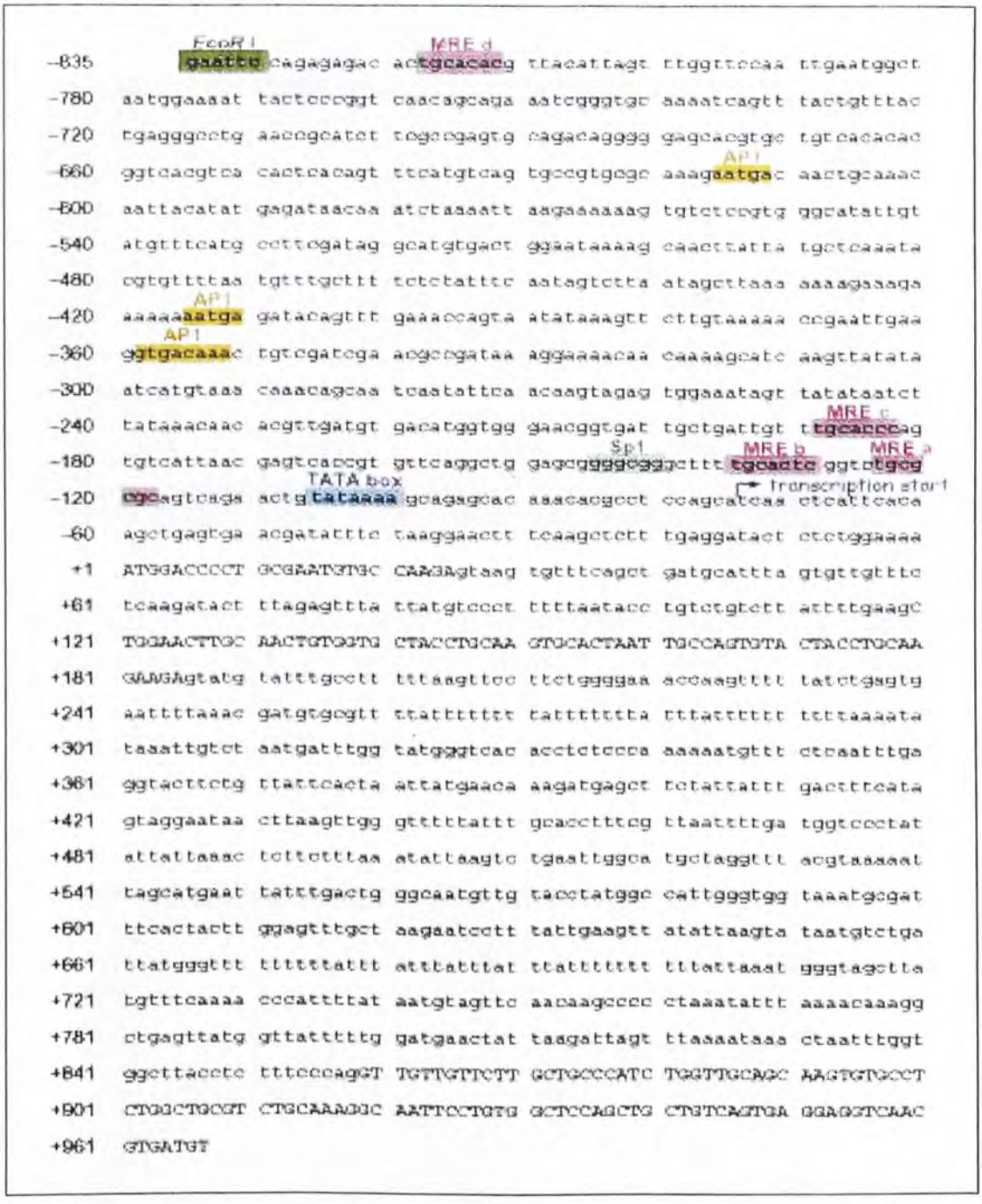



Fig. 1-6 The nucleotide sequence of the zebrafish MT gene. The translation start site is designated as +1 and the putative transcription factor binding sites are indicated with different colours (Yan and Chan, 2004).

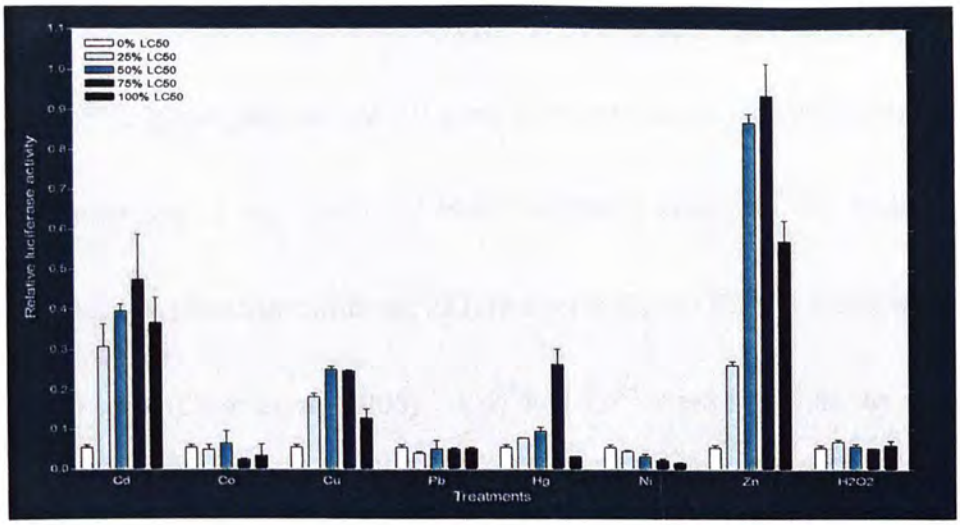



Fig. 1-7 Transcriptional levels *zMT*/F1-Luc constructs transfected in *HepG2* cells after the administrations of various metal treatments. The transfected cells were treated with $Cd^{2+}$, $Cu^{2+}$, $Pb^{2+}$, $Hg^{2+}$, $Ni^{2+}$, $Zn^{2+}$ and $H_2O_2$ at different dosages (0%, 25%, 50%, 75% and 100% of their 24h-LC50 values). Each value represents the mean ± S.D. of three replicates . (Yan and Chan, 2004)

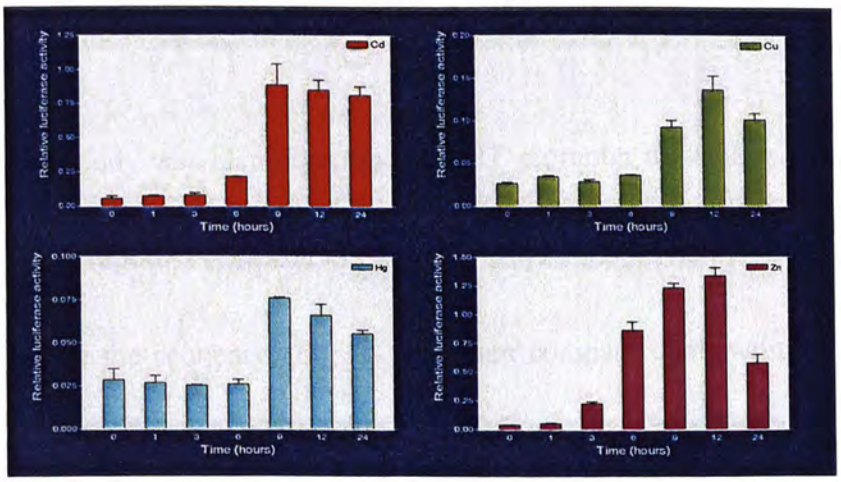



Fig. 1-8 Time course study for treatments of $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$, and $Zn^{2+}$ on *HepG2* cells transfected with *zMT*/F1-Luc construct. Metal concentrations that caused maximal zMT induction were used in the time course study. Each value represent the mean ± S.D. of three replicates. (Yan and Chan, 2004)

zMT gene promoter induction as the metal ions mentioned previously.

To further investigate the zMT-II gene promoter using fish cell-lines, the zMT-II gene promoter cloned into the pGL3-Basic luciferase construct was transfected into two homologous zebrafish cell lines: ZFL (a liver cell) and SJD (a fibroblast cell from caudal fin) cells (Chan *et al.*, 2006). $Cd^{2+}$ and $Zn^{2+}$ were found as the most potent inducers in activating the zMT promoter in both cell lines while $Hg^{+}$ is a moderate inducer. For the results of $H_2O_2$, $Ni^{2+}$, $Pb^{2+}$ and $Co^{2+}$, no significant induction of zMT-II romoter activity was observed (Fig. 1-9 and Fig.1-10). Comparing with the study in HepG2 cell line (Yan and Chan, 2004), the two studies showed a similar induction pattern of zMT promoter.

Similar study was also done on the zMT promoter as well, where a 1.4kb long promoter was reported (Chen *et al.*, 2004). Seven MREs named as MRE1 to MRE7 were found in the sequence (Fig. 1-11), when compare them with the MREs in the 0.8kb zMT promoter sequence (Fig. 1-11), MREa, MREb, MREc and MREd are equal to MRE1, MRE2, MRE4 and MRE6 respectively. The sequence of MRE3 in the 1.4kb zMT promoter is GTGTGCA (5'-TGCACAC-3'), meanwhile similar sequence is present in the exact position in the 0.8kb zMT promoter, which is

GTGTTCA (5'-TGAACAC), but it is not matched with the MRE core sequence (5'-TGCRCNC-3'). MRE5 is actually present in the 0.8kb zMT promoter but it's ignored in the publication, its position is shown with green color. The MRE7 is absent in the 0.8kb zMT promoter, because it's located in the position that beyond the length of the sequence.

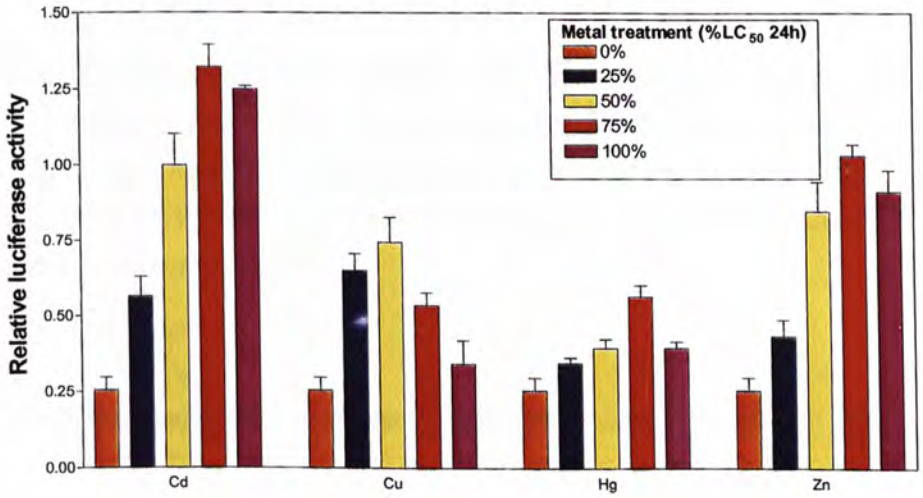



Fig. 1-9 Transcription levels study in *ZFL* cells, by transfecting the cells with *zMT*-F1-luc construct followed by various metal treatments. Transfected cells were treated with $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Zn^{2+}$ in the dosages corresponding to 0% 25%, 50%, 75% and 100% of their LC50 values for 24 h. Each value represents the mean of ± S.D. of three replicates (Chan *et al.*, 2006).

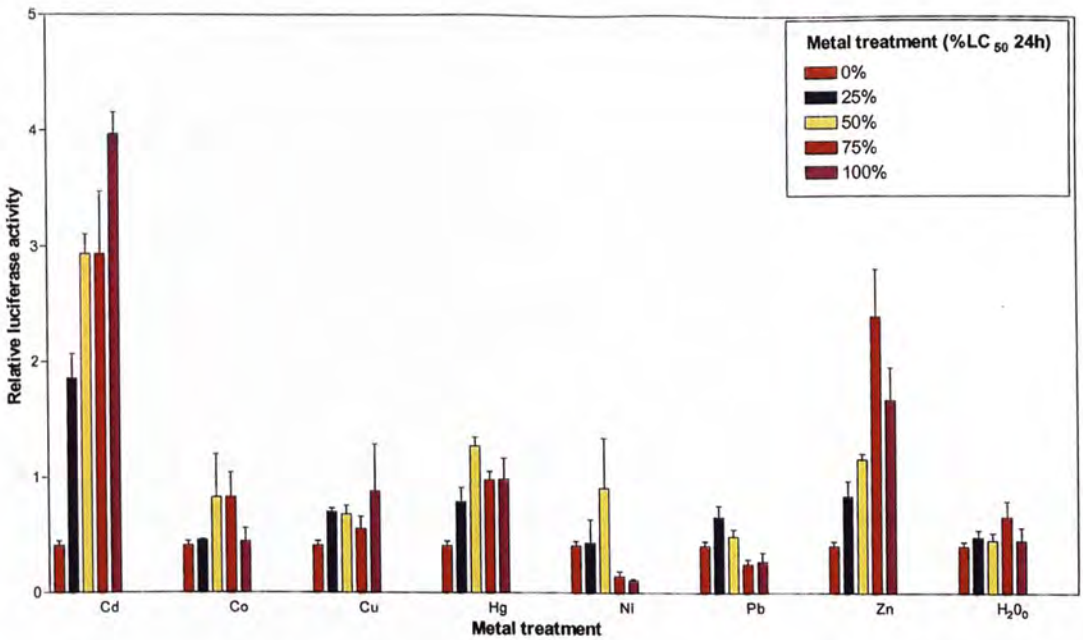



Fig. 1-10 Transcription levels study in *SJD* cells, by transfecting the cells with *zMT*-F1-luc construct after various metal treatments. Transfected cells were treated with $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Zn^{2+}$ in the dosages corresponding to 0% 25%, 50%, 75% and 100% of their LC50 values for 24 h. Each value represents the mean of ± S.D. of three replicates (Chan *et al.*, 2006).

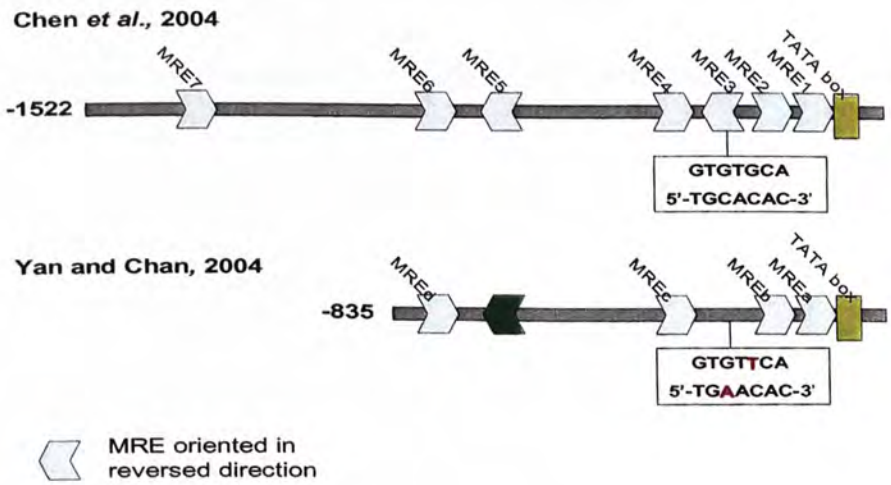



Fig. 1-11 Schematic diagram showing the structures of the 1.4kb and 0.8kb zMT-II promoters (Chen *et al.*, 2004; Yan and Chan, 2004). MRE3 and MRE7 are absent in the 0.8kb zMT-II promoter gene. For MRE5 (MRE shown in green color), it is actually present in the 0.8 kb sequence but ignored in the publication.

As mentioned, two MT isoforms, MT-A and MT-II, are present in the zebrafish. The amino acid sequence of the 0.8kb and 1.4kb sequences were exactly the same, therefore it is believed that the two sequences are referring to the same zMT-II promoter or zMT-IIa and zMT-II b gene promoters.

Chen *et al.*(2004), studied the promoter activity by conducting transient luciferase gene expression assay in Hep3B cell line, the promoter was activated after the transfected cells were exposed into 200 μM of $ZnSO_4$ for 7 hr. (Fig. 1-12; Chen *et al.*, 2004). This study showed that $Zn^{2+}$ was able to cause induction in the 1.4kb zMT promoter activity in Hep3B cell line, however the effects of other metal ions on the activities of this promoter in zebrafish cell lines remain to be investigated.

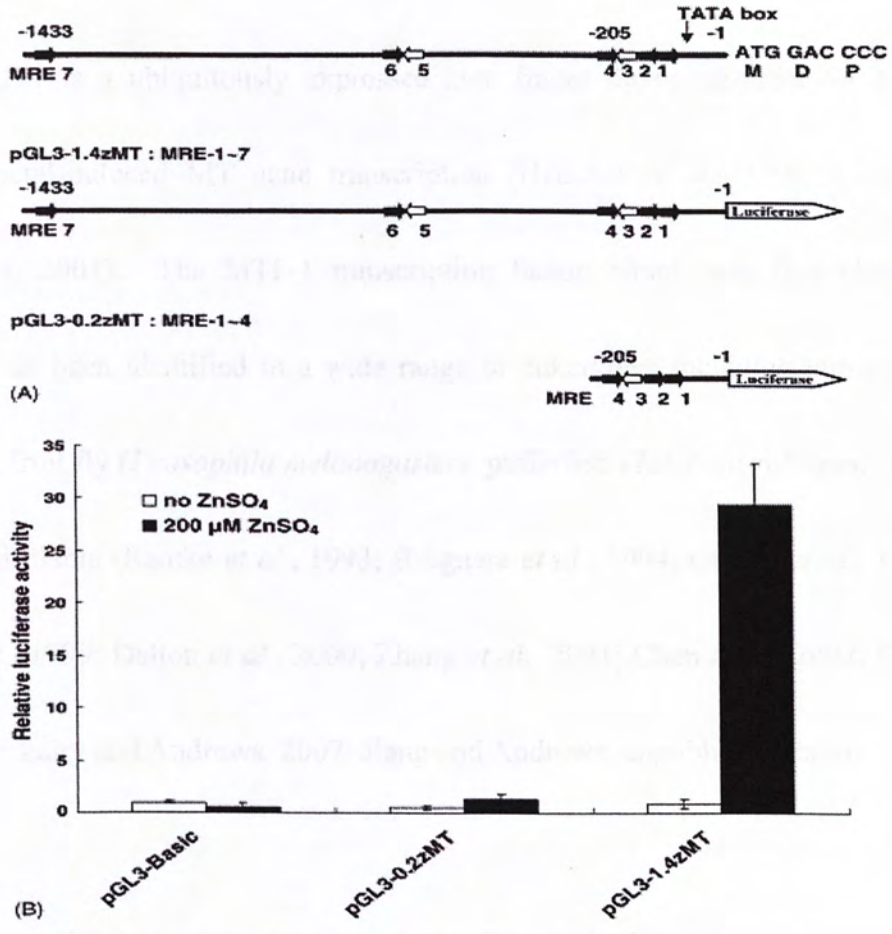



Fig. 1-12 Transcriptional level study on *Hep 3B* cell line. Cells transfected with pGL3-1.4zMT construct shown induction in relative luciferase activity after treating with 200μl $ZnSO_4$ for 7 hours (adapted from Chen *et al.*, 2004).

## 1.6 MRE-Binding Transcription Factor-1 (MTF-1)

### 1.6.1 Structure of MTF-1

MTF-1 is a ubiquitously expressed zinc finger factor essential for basal and heavy metal-induced MT gene transcription (Heuchel *et al.*, 1994; Lichtlen and Schaffner, 2001). The MTF-1 transcription factor, which was first cloned from mouse, has been identified in a wide range of eukaryotes including human, mouse, chicken, fruit fly (*Drosophila melanogaster)*, pufferfish (*Takifugu rubripes)*, zebrafish, trout and tilapia (Radtke *et al.*, 1993; Brugnera *et al.*, 1994; Otsuka *et al.*, 1994; Auf der *et al.*, 1999; Dalton *et al.*, 2000; Zhang *et al.*, 2001; Chen *et al.*, 2002; Cheung *et al*, 2004; Laity and Andrews, 2007; Jiang and Andrews, unpublished data).

The MTF-1 protein is around 80kDa, including at least three distinct transcriptional activation domains with high contents of acidic and proline residues located in the C-terminal half of the molecule with a six $Cys_2$-$His_2$ zinc finger domain located at the N-terminus of the molecule (Radtke *et al.*, 1993; Chen *et al.*, 1998). Similar to the MRE, MTF-1 is conserved in evolution, the human, mouse and fish and their homologues are highly similar with a 93% amino acid identity (Brugnera *et al.*, 1994; Lichtlen and Schaffner, 2007) and the DNA binding zinc fingers are most strictly conserved (Brugnera *et al.*, 1994)

Fig. 1-13 is a schematic diagram of the structures of MTFs obtained from different animal species. A MTF-1 of zebrafish was cloned and its cDNA sequence published (Chen *et al.* 2002, GeneBank number: AF458116). It is 3379 bp in length, encoding for one open reading frame of a polypeptide of 593 amino acids. The zebrafish MTF-1 consists of six zinc finger domains at the N terminal and three transcriptional activation domains in the C terminal and it shows high identity of 97 %, 93 %, 93 % and 67% in the zinc finger domain and 51 %, 44 %, 48 % and 20% overall identity with fugu, human, mouse and *Drosophilia*, respectively (Chen *et al.*, 2002)

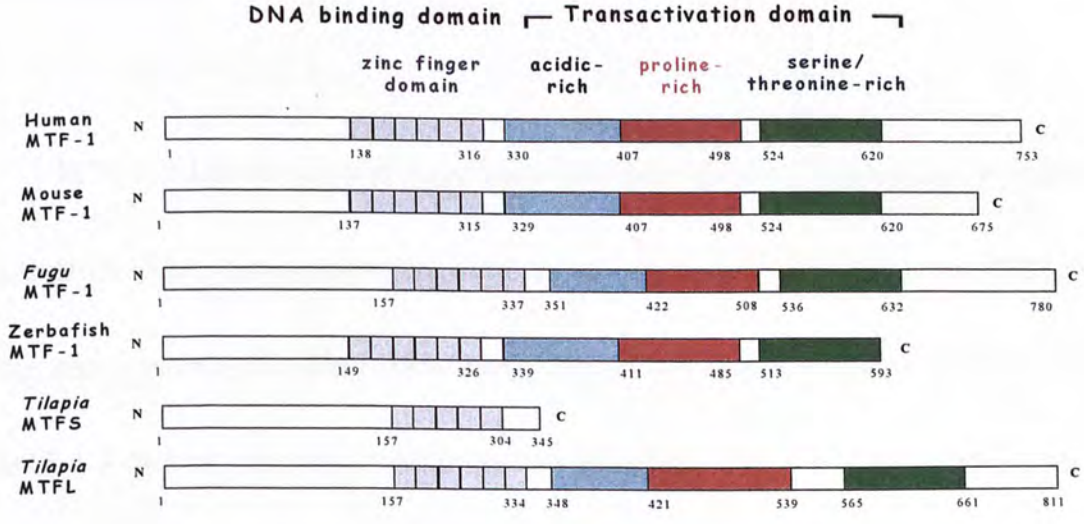



Fig. 1-13 Schematic diagram showing the DNA binding domain and transactivation domains of human, mouse, pufferfish, tilapia and zebrafish MTF-1 (Brugnera, *et al.*, 1994; Radtke, *et al.*, 1993; Auf der Maur, *et al.*, 1999; Chen, *et al.*, 2002).

### 1.6.2 Physiological functions of MTF-1

MTF-1 has several physiological functions, previous studies indicated that MTF-1 knockout mice embryos die in utero around embryonic day 13-14 of gestation due to degeneration of the embryonic hepatocytes, therefore MTF-1 is believed to be essential for the normal liver development. Although MTF-1 can be eliminated in adulthood of the mice, they become more susceptible to heavy metal stress once the MTF-1 is eliminated (Gunes *et al.*, 1998; Lichtlen and Schaffner, 2001; Lichtlen and Schaffner, 2007). Similar result was obtained in *Dorsophila*, knocking out of MTF-1 in *Dorsophila* made them more sensitive to cadmium, copper and zinc toxicities (Egli *et al.*, 2003).

MTF-1 is known to be an important component for the basal and metal induced activation of MT gene expression, however MT is not the sole target gene of MTF-1, this was proven by the observation that MT-I and MT-II-null mouse was viable while MTF-1 knockout was lethal (Lichtlen and Schaffner, 2001). $\gamma$ -glutamyl cysteine synthetase is another target gene of MTF-1, it is for producing gluthathionein (GSH) to protect cells against oxidative stress and heavy metal toxicity in a less specific way by binding to metal ions (Meister, 1995; Gunes *et al.*, 1998). MTF-1 also mediates metal regulation of zinc transporter-1 (ZnT1) which helps in the regulation of zinc and

cadmium ions levels in the cells (Langmade *et al.*, 2000). As these three target genes are deal with the heavy metal homeostasis and oxidative stress, MTF-1 is a protein that helps in coping with stress.

### 1.6.3 The role of MTF-1 in MT gene regulation

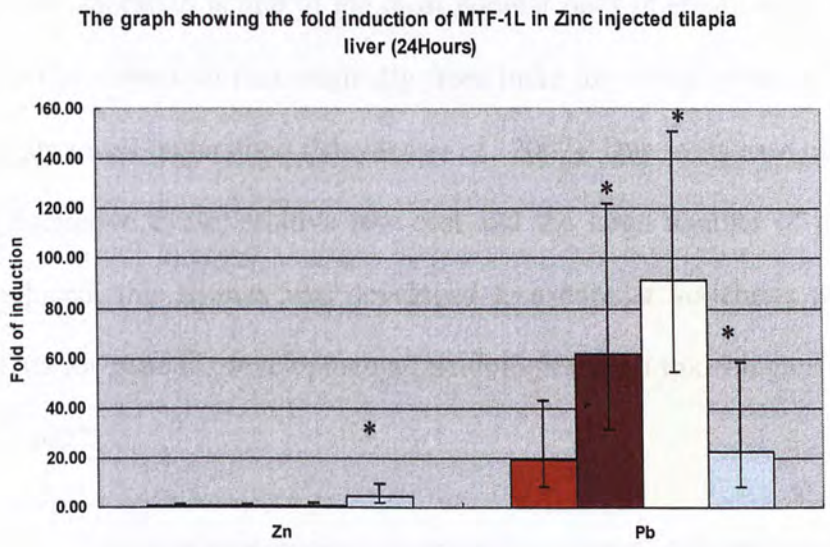
MTF-1 is a *trans*-acting element that can trigger the MT gene by binding to the specific target sequence of DNA in the promoter region, therefore it's an important component for the MT gene expression. Unlike other zinc finger transcription factors, which appear to be constitutively active to bind DNA under normal physiological conditions, MTF-1 is reversibly bind to DNA and the binding is depended on changes of zinc availability (Westin and Schaffner, 1988; Radtke *et al.*, 1993; Dalton *et al.*, 1997; Koizumi *et al*, 1999; Bittel *et al.*, 2000). In other words, the binding of MTF-1 to the MREs requires activation by zinc, as proposed by the zinc-pool hypothesis. Once the MTF-1 sense free zinc ion, it adopts a reversible DNA-binding conformation, this allosteric change causes exposure of zinc fingers. Then the MTF-1 moves to the nucleus, and activates gene expression by associating with the promoter (Andrews, 2001; Lichtlen and Schaffner, 2001; Jiang *et al.*, 2003).

### 1.6.4 Regulation of MTF-1 by various heavy metals

Because MTF-1 is the protein that mediates the induction of MT gene expression, it is interesting to know the mechanism of this process. Previous studies pointed out that the mediation of MT gene expression is begun by the translocation of MTF-1 protein (Smirnova *et al.*, 2000; Chen *et al.*, 2007). MTF-1 is originally located in the cytoplasm, heavy metals like cadmium and zinc ions mediate the translocation of MTF-1 to the nucleus, and the protein will then bind to MREs so that triggers the activation and consequently expression of MT gene. However, the increase in MTF-1 and MREs binding does not seem to be merely due to the increase of nuclear MTF-1 but also due to the increase of MTF-1 content in the cell.

Some study of MTF-1 was done on tissues of Tilapia (Cheung, 2003). Unlike other species, Tilapia has two different forms of MTF-1, the long form (MTF-1L, 2598 bp) and the short form (MTF-1S, 1647 bp), where MTF-1L consists of six zinc fingers and three transcription activation domains and MTF-1S consists of five zinc fingers only and it does not have the three transcription activation domains (Cheung, 2003; Fig. 1-13). Tilapia was injected with sub-lethal doses of zinc and lead ion solution, real time PCR was preformed to quantify the mRNA level of two different forms of MTF-1 in the liver of Tilapia after 24 h (Chan, unpublished data). The

results indicated that $Zn^{2+}$ is able to induce MTF-1 long while lead is able to cause induction to the MTF-1L as well as the MTF-1S. (Fig. 1-14). Therefore it is possible that heavy metals ion exposure can activate the production of MTF-1 protein.



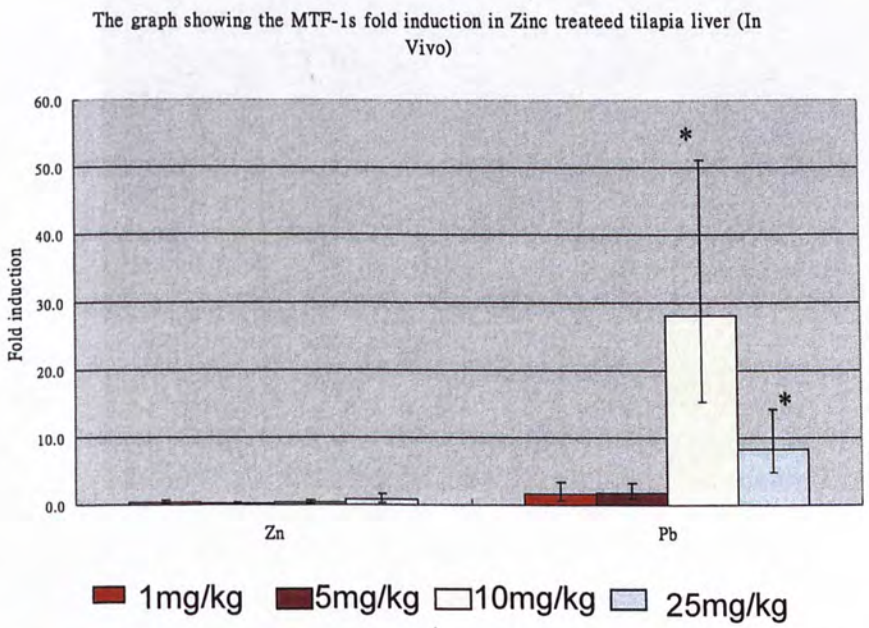




Fig. 1-14 (A) MTF-1L and MTF-1S content in the liver of the *Tilapia* was studied by the real-time PCR after injected with $Zn^{2+}$ and $Pb^{2+}$, significant MTF-1L mRNA inductions were observed after $Zn^{2+}$ and $Pb^{2+}$ injection. However, only $Pb^{2+}$ injection would cause induction of MTF-1S mRNA in the *Tilapia* liver.

## 1.7 Zebrafish (*Daino reio*)

Fish is a useful model to study the effects of metal ions on the MT gene regulation because they are living in the aquatic environment and so have more chance to contact with the metal ion (Chan, 1995). And among the different species of fish, zebrafish is one of the most popular ones internationally. Zebrafish (*Danio rerio*) is a cyprinid fish originally from India for which genetics and nervous system are also well understood (Mayden *et al.*, 2007). Due to its ease of maintenance, short reproductive cycle, relative low cost and the large number of progeny that can be produced, this species was developed to a popular vertebrate model system at the 1970s for genetic, developmental, neurological, and toxicological studies (Mayden *et al.*, 2007).

Fig. 1-15 Zebrafish (*Danio rerio*) (adapted from http://www.cas.vanderbilt.edu/bioimages/animals/danrer/zfish-devel.htm)

## 1.8 Project aim

MT gene expression is inducible by heavy metal ions and has been shown to play an important role in the sequestration and detoxification of heavy metal ions. Since MT mRNA is inducible by heavy metals, its quantification can be useful biomarker to evaluate the exposure histories to heavy metal pollution. However, differential inductions of MT mRNA levels by different metal ions remain to be tested in each fish species. In this regard, we are using zebrafish MT gene expression as a model to evaluate the effects of different metal ions on MT gene expression *in vivo* and *in vitro*.

The advantages of the zebrafish adult and embryo-larval models provide researchers with an ideal *in vivo* platform to elucidate the molecular link between the heavy metal ions and MT gene regulation for toxicological testing. Preliminary studies reported that zebrafish embryo-larva is a sensitive model for exposure experiments and could accumulate metal ions (Li *et al.*, 2004; Chan *et al.*, 2006).

A zMT-II gene was obtained aiming at understanding the gene structure and organization of the promoter region using zebrafish as a model (Yan and Chan, 2002 and 2004). The gene promoter region of 835 bp with four MREs was transfected

into SJD (zebrafish caudal fin fibroblast cell) (Yan and Chan, 2002), and HepG2 cells (human hepatic cell-line)(Yan and Chan, 2004) for transient gene expression studies following the administration of different metal ions. Inductions of zMT-II promoter after administrations of $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$ and $Hg^{2+}$ were confirmed in decreasing potency, while hydrogen peroxide, $Ni^{2+}$, $Pb^{2+}$ and $Co^{2+}$ did not induce any significant fold induction of the zMT gene promoter in HepG2 and SJD cells (Yan and Chan, 2002 and 2004). However, HepG2 is a human hepatocyte cell-line and a homologous hepatic system would provide more accurate analyses of the cloned zMT gene promoter, while SJD is a fibroblast cell-line from zebrafish. Chen *et al* (2004) also identified a similar zMT gene with further upstream region containing 7 MREs but only induction by $Zn^{2+}$ and developmental regulation was studied. Hence, a longer MT promoter gene with more MREs should be characterized in zebrafish cell-lines in order to obtain a clear picture of how different metal ions activate zMT gene transcription *in vitro*.

The aim of this project is to investigate the induction of zMT gene expression following the administration of different metal ions using *in vitro* models of zebrafish. We use zebrafish cell-lines, ZFL (zebrafish liver cell) and SJD (zebrafish caudal fin fibroblast cell), to characterize the zMT gene regulation caused by different heavy

metals ($Zn^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Hg^{2+}$, $As^{3+}$, $As^{5+}$, $Cr^{3+}$ and $Cr^{6+}$). The inductions of MTF-I and zMT mRNA levels by different heavy metals as well as zMT gene promoter levels were studied. This helps in comparing the toxicities of different metal ions and mechanisms involved in zMT gene activation via the MTF-1 pathway in response to different metal ions in the two cell lines. The information collected will be useful to delineating how different metal ions activate MT gene expression and evaluating the use of zMT gene as a biomarker of metal exposures, using zebrafish cell-lines for bio-monitoring of metal contamination in the aquatic environment.

# Chapter 2

## Materials and Methods

### 2.1 Cell culture

#### 2.1.1 ZFL cell line

ZFL cells are zebrafish liver cells obtained from American Type Culture Collection (ATCC, CRL-2643), these cells were maintain in a 28 ℃ incubator, and cultured with 50% Leibovitz's L-15 medium with 2 mM L-glutamine (Vitacell 30-2008), 35% Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and 4 mM L-glutamine (Gibco 12100), 15% Ham's F12 with 1 mM L-glutamine (Gibco 21700), 15 mM HEPES, 0.01 mg/ml insulin (Sigma I-1882), 50 ng/ml epidermal growth factor (EGF), 0.15 g/L sodium bicarbonate, and 5% heat-inactivated fetal bovine serum (FBS) was freshly added before culture started.

The cells were sub-cultured by removing the medium, rinsing with PBS (phosphate buffer saline) and then added with 1-2 ml trypsin (0.25%)-EDTA (0.03%)-polyvinylpyrrolidone (0.5%) solution. The flask was placed at room temperature until all cells were detached. 2-3 ml medium was added and the cells were subjected to centrifugation at 150 g for 5 min. Cell pellet was resuspended in serum-free medium, 1/3 of cells was added into the new flask which contained serum-free medium. The culture was incubated at 28 ℃ incubator for 30 min to let

the cells attach, heat-inactivated FBS was then added at 5% of total volume.

### 2.1.2 SJD.1 cell line

SJD.1 is the caudal fins cells of the zebrafish (ATCC# CRL-2296) cultured at a 28 ℃ incubator with 5% $CO_2$, in the medium of DMEM with 4 mM L-glutamine and 4.5 g/L glucose (Gibco 12100), and 15% heat-inactivated FBS. The cells were sub-cultured by removing the medium and then rinsed with phosphate buffered saline; 1-2 ml of trypsin solution was added after the rinsing. The culture was placed at room temperature until all cells detached, fresh medium was then added and the cells were collected by centrifugation at 150 g for 5 min. The cells pellet was resuspended with fresh medium and 1/3 of them were added into a new flask.

## 2.2 Alarmar blue ™ assay

To obtain toxicity reference data for metal exposure study, Alarmar blue ™ assay was conducted to determine the $EC_{50}$ values of different metal ions on the two zebrafish cell lines. Viable cell count was performed with Trypan Blue exclusion assay to determine the cell numbers in culture. Cells ($1 \times 10^5$) were seeded in each well of the flat-bottomed 96-well plates and incubated for one night; metal solutions were diluted serially with the culture medium to different concentrations and added to

the cultured cells. Six replicates were done on the same plate for each dosage of metal treatments; the treated cells were incubated in the incubator.

After 24 hr, the medium was replaced with fresh medium containing alarmarBlue™ amounted to 10% of total medium volume, and incubated for 5 hr. Fluorescence readings from each well were measured by CytoFloue™ 2350 Fluorescence Measurement System (Millopore) with excitation wavelength at 485 nm and emission wavelength at 595nm. The $LC_{50}$ values of different heavy metal ions to the cells were determined by using the GrandPad Prism® analysis software on a personal computer.

### 2.3 First strand cDNA synthesis

#### 2.3.1 Metal treatment of the SJD and ZFL cell lines

Cells ($8 \times 10^5$) were seeded in each well of a 6-well culture plate and stand for one night. After the incubation, metal solutions were diluted serially with the culture medium to 10%, 25%, 50%, 75% and 100% of their 24h-$EC_{50}$ values. The medium in the plate was replaced with different concentrations of metal solution. Six replicates were done on the six plates for each dosage of different metal treatments; the treated cells were incubated in the incubator. Cells used in MT study were

treated with heavy metal solution for 24 h while the treatment duration of the cells for MTF-1 study was 12 h.

### 2.3.2 Isolation of total RNA

The cells treated with heavy metal ions were washed with PBS and homogenized with 0.6 ml of Tri-Pure Isolation Reagent (Roche, Indianapolis, IN, USA) on ice, 100 µl 1-bromo-3-chloropropane (BCP) was added after homogenization and mix thoroughly. After centrifugation at 12,000 Xg for 15 min at 4℃ to separate the organic phase and aqueous phase, the upper aqueous phase was transferred into a fresh tube and then added with 500 µl isopropanol to precipitate total RNA. The tubes were then centrifuged at 12,000 Xg for 15 min at 4℃ to form RNA pellets. The RNA pellets were washed with 500 µl 70% ethanol, and allowed to air dry. Finally, the RNA pellets were dissolved in 40 µl DEPC-treated dd$H_2O$ at 70 ℃ for 10 min and the dissolved RNA samples were stored at -80 ℃.

### 2.3.3 Quantification of mRNA by spectrophotometer

Two µl of the RNA samples was added into 498 µl d$H_2O$ and mixed thoroughly. The diluted RNA samples were measured at absorbance in 260 nm and 280 nm using a quartz cuvette at 1 cm path to obtain their $OD_{260}$ and $OD_{280}$ values by using a

Hitachi U-2800 spectrophotometer. The $OD_{260}$ value represents RNA concentration of 10 μg/μl and the RNA with high purity should have a ratio of $OD_{260}/OD_{280}$ value around 2.0.

### 2.3.4 Reverse transcription

One μg of RNA was incubated with 100 ng random primer at 70 ℃ for 5 min followed by 4 ℃ for 5 min for annealing of primer to RNA. A mixture of 1 mM dNTP, 2.0 mM $MgCl_2$, 1 μl enzyme, and 4 μl reaction buffer was added into each sample on ice and incubated at 25 ℃ for 5 minutes, followed by incubation at 42 ℃ for 60 min for cDNA synthesis and 72 ℃ for 15 min for inactivation of enzyme activity. The first strand cDNA sample was stored at - 38℃ until use.

## 2.4 Quantifications of mRNA levels by using real-time PCR technique

### 2.4.1 Primer design

Primers for zebrafish β-actin, MT and MTF-1 genes were designed based on the sequences published in GeneBank (Zebrafish β-actin, GenBank number: BC_045879; Zebrafish MT, GenBank number: NM_194273; Zebrafish MTF-1, GenBank number: AF458116). Using the ABI Primer Express Program (PE-Applied Biosystems, Forster City, CA, USA) following the manufacturer's instruction, specific primers (Table 2-1) were designed spanning over two exons at the intro-exon junction sites to

Table 2-1 Nucleotide sequences of gene specific primers for real-time PCR of zebrafish β-actin, zMT and MTF-1.

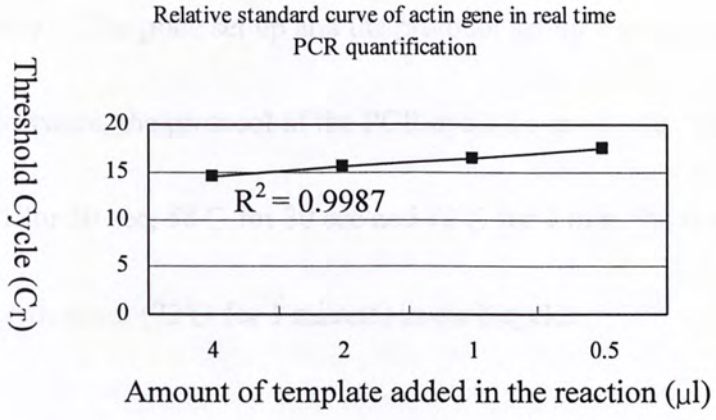
| Target gene | Primer | Nucleotide sequence (5" to 3') | Amplicon size |
|---|---|---|---|
| *B-actin* | Forward Primer | CTT GGG TAT GGA ATC TTG CG | 88 bp |
| | Reverse Primer | AGC ATT TGC GGT GGA CGA T | |
| *ZMT* | Forward Primer | GCC AAG ACT GGA ACT TGC AAC | 130 bp |
| | Reverse Primer | CGC AGC CAG AGG CAC ACT | |
| *MTF-1* | Forward Primer | TCA GTG ACC TGA GGA AGC AC | 98 bp |
| | Reverse Primer | GGT GAT GAC TAG CAG CGA AA | |

avoid the primers binding on the genomic DNA sequences and amplify contaminated DNA in the samples, and hence genomic contamination in RT-PCR could be minimized. The program also checked for avoidance of hair-pin formation and optimization of small amplicon sizes for efficient amplification.

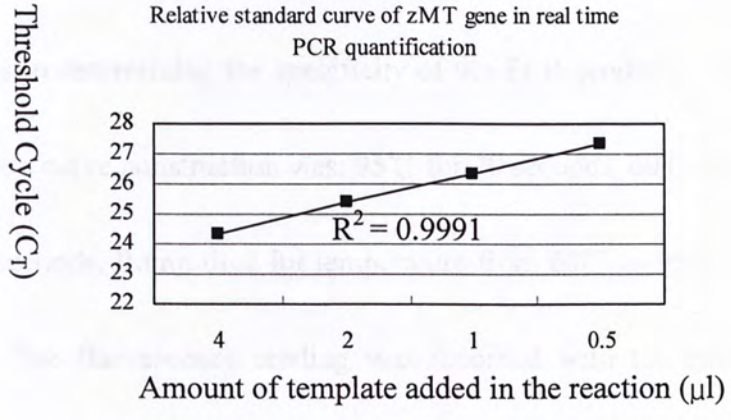
### 2.4.2 PCR components and cycling condition

To perform real-time PCR, reaction mixture was set up in a 0.2 ml clear thin-walled, optical grade PCR tubes, which contained 12.5 μl Brilliant® SYBR® Green QPCR Master Mix (ABI), 0.5 μl of forward primer (10 μM), 0.5 μl of reverse primer (10 μM), 2 μl cDNA template and 9.5 μl nuclease-free water. Standard curve (Fig. 2-1) was constructed by adding 4, 2, 1 and 0.5 μl of templates into the reaction

(A)




(B)




(C)

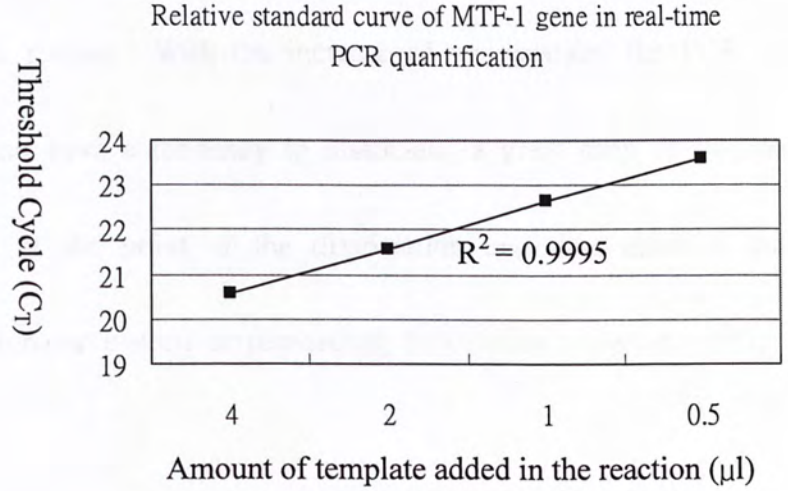


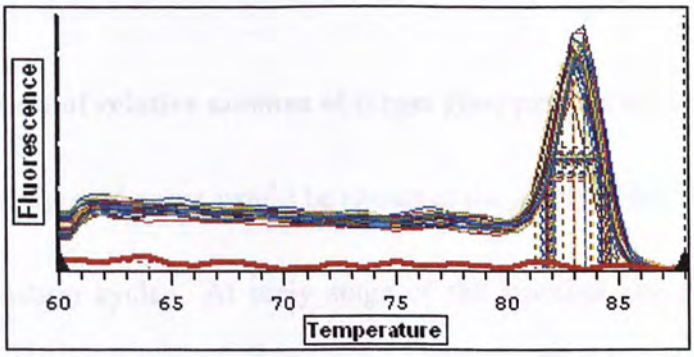

Fig.2-1 Relative standard curves for (A) actin gene, (B) zMT gene and (C) MTF-1 gene in real-time PCR quantification. The linear relationship ($R^2 = 0.99$) indicated that $C_t$ is inversely proportional to the initial amount of the target template.

mix respectively. The plate set up and the protocol set up was done with the Opticon Monitor 3.0 software, the protocol of the PCR cycle is: one cycle 95℃ for 10 min, 40 cycles at 95℃ for 30 sec, 58℃ for 30 sec and 72℃ for 1 min, the fluorescence was read after the extension (72℃ for 1 minute) in each cycle.

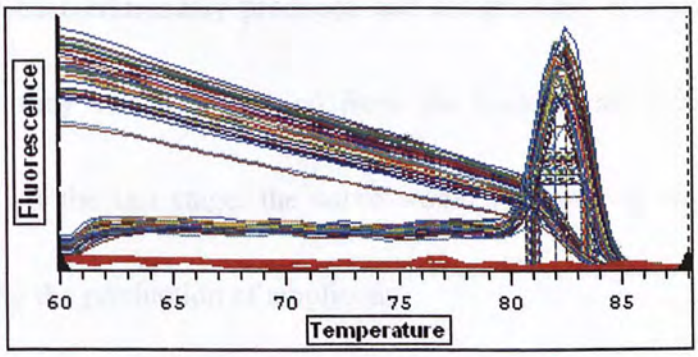
Dissociation curve analysis was set after the 40 cycles of amplification, this analysis helps in determining the specificity of the PCR product. The condition for the dissociation curve construction was: 95℃ for 20 seconds, 60℃ for 15 seconds and 95℃ for 20 seconds, Ramp time for temperature from 60℃ to 95℃ would be set for 20 minute. The fluorescence reading was recorded with the gradual increase in temperature, and the result of fluorescence change △Rn against temperature (℃) would be shown. With the increase of temperature, the PCR products gain the energy and have a tendency to dissociate, a great drop of fluorescence would be appeared at the point of the dissociation and thus cause a sharp peak in the dissociation curve at the corresponding dissociation temperature (Fig. 2-2).

Each gene should have its own specific dissociation temperature; appearance of two or more peaks indicates PCR product(s) other than target gene was amplified. In other words, the reaction mixture was contaminated. A no template control (NTC) should be set in each set of real-time PCR experiment; contamination can be detected

(A)




(B)




(C)

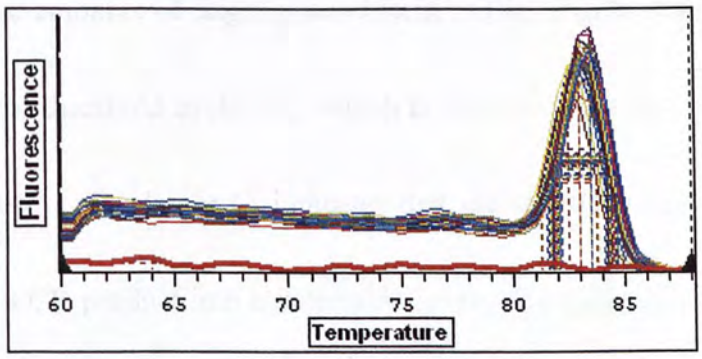



Fig. 2-2 (A) The dissociation curve of the actin gene, with the dissociation temperature of 82.5 ℃. The dissociation curve of the NTC was indicated as the thick red line. (B) The dissociation curve of the MT gene, with the dissociation temperature of 83 ℃. The dissociation curve of the NTC was indicated as the thick red line. (C) The dissociation curve of the MT gene, with the dissociation temperature of 82 ℃. The dissociation curve of the NTC was indicated as the thick red line.

if there is any peak appears in the dissociation curve of the NTC.

### 2.4.3 Determination of relative amount of target gene present in the samples

After PCR, a sigmoid curve would be shown at the graph of fluorescence change ($\triangle$R) against reaction cycle. At early stage of the reaction, the amount of PCR product is too little to distinguish from the background noise level. But the PCR products would be continuously produced and accumulated at a point of threshold level after that they can be identified from the background noise and increased exponentially. At the last stage, the curve would be level off as the depletion of reagents, limiting the production of amplicons.

The relative amounts of target gene present in the samples are calculated based on the concept of threshold cycle ($C_t$), which is the cycle that the reaction begins its exponential phase. The larger $C_t$ indicates that the sample requires more cycle to accumulate the PCR product into a detectable level and begins its exponential phase, which means that the initial amount of target gene present in the sample is relatively less than the samples that having a smaller $C_t$.

The $C_t$ of the target gene in the samples should be normalized with the $C_t$ of the

house keeping gene (β-actin) by finding their difference ($C_t$ of actin - $C_t$ of zMT), where the result is $\triangle C_t$. The fold induction of zMT expression in the metal treated sample to the control sample can be calculated by the formular:

$$2^{(\triangle Ct \text{ of control sample} - \triangle Ct \text{ of treated sample})}$$

**2.5 Cloning of zMT-II gene promoter and its transient expression studies**

**2.5.1 Purification of genomic DNA**

An adult Zebrafish of local strain was obtained from the local market at Chong Hing Aqurium Company) and homogenized in 80 ml Grinding Buffer (0.1 M Tris, 0.05 M $Na_2EDTA$, 0.2 M NaCl, 1% SDS) with a mortar, with the presence of liquid nitrogen. Proteins were digested by overnight incubation at 65 ℃ with Proteinase K (Boehringer Mannheim), and then followed by phenol-chloroform extractions. Five M NaCl was added in 0.2 volumes and 2 volumes of absolute ethanol were added to precipitate the genomic DNA.

The precipitated DNA was washed with 70% ethanol and dissolved in $dH_2O$ with 10 mM Tris-HCl, pH 8.0, and 5 mM EDTA. The genomic DNA samples were measured at $OD_{260}$ and $OD_{280}$ by using the U-2800 Hitachi spectrophotometer, the ratio of the absorbance were calculated in order to determine the purity and quantity

of samples obtained as mentioned in 2.3.3, except that 5μl of the DNA samples was added into 995 μl of $dH_2O$.

### 2.5.2 Preparation of Escherichia coli competent cell

Single colony of *E. coli* from frozen stock (strain DH5α) was inoculated in CG medium (Molecular Biology Certified Bacterial Growth Medium, BIO 101, Inc) until $OD_{600}$ reached the absorbance within the range of 0.25 to 0.4. Cells were chilled on ice for 5 mins and collected by centrifugation at 6000 rpm for 10 min at 4 ℃. Ice-cold filter-sterile $Ca^{2+}$/glycerol buffer (60 mM $CaCl_2$, 10 mM Pipes, 15% glycerol) was used to dissolve the cell pellet. Cells were centrifuged again at 6,000 rpm for 10 min at 4 ℃, and gently resuspended by ice-cold sterile filtered $Ca^{2+}$/glycerol buffer and further incubated on ice for 30 min. Cells were collected by centrifugation at 6,000 rpm for 5 min at 4 ℃, gently resuspended in only 5 ml of $Ca^{2+}$/ glycerol buffer, dispensed into 100 μl aliquots, frozen immediately in liquid nitrogen and then stored at -80 ℃ until use.

### 2.5.3 PCR-Cloning of a 1.4 kb zMT-II gene promoter

Primers were designed based on the sequence published in Gene bank (accession number: AY514791).

| Primer | Sequence (5' to 3') |
|---|---|
| zMT-II-Forward | TCCTCAACACTGATTAGTCTAC |
| zMT-II-Reverse | CCAGAGAGTATCCACAAAGAG |

The 1.4kb zMT-II promoter was amplified by genomic PCR reaction Expand High Fidelity PCR System (Roche Applied Science). Purified zebrafish genomic DNA (200 ng) was mixed with 5 μl of 10X Expand High Fidelity buffer, 15 mM $MgCl_2$, 1.5μl of 10 μM zMT-Forward primer, 1.5 μl of 10 μM zMT-Reverse primer, 1 μl of 10 mM of dNTP mix, 0.75 μl of Expand High Fidelity Enzyme Mix and distilled water was finally added to make up the reaction volumn to 50 μl.. The PCR reaction was performed on a TaKaRa PCR Thermal Cycler "Dice" with the following PCR conditions: one cycle 94 ℃ for 2 min, 25 cycles at 94 ℃ for 40 sec, 58 ℃ for 40 sec and 72 ℃ for 3 min, followed by one cycle of 72 ℃ for 7 min.

The PCR product was resolved in a 0.8% agarose gel and visualized under UV illumination after staining with ethidium bromide. The stained DNA band was extracted and purified with CONCERT™ Rapid Gel Extraction System (GibcoBRL) according to the manufacturer's instruction. The purified product was cloned into pGL3-Basic Luciferase Reporter Vector (Promega, Madison, WI, USA) at *Kpn*I and *Hind*III sites according to the procedures in the manual, and the plasmid was then used to transform the *E. coli* (DH5α strain) competent cells. The clones were

screened by the blue-white screening followed by PCR reaction with GLprimer2 and RVprimer3. The plasmid DNA of the positive clones was prepared by QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. The purified plasmid DNA was then sequenced with GLprimer2 and RVprimer3.

### 2.5.4 Purification of plasmid DNA

*E. coli* competent cells (DH5α) were thawed on ice and simultaneously the purified plasmid DNA was mixed in, the mixture was left on ice for 5 minutes and then heated at 42 ℃ for 90 sec for a heat shock and then placed on ice for 5 min. Sterile CG medium was added and the samples were incubated at 37 ℃ for 45 min for recovery. The whole sample in CG medium was poured on agar plate in LB medium with ampicillin (100 mg/L). After overnight incubation at 37 ℃, the colonies were checked by digesting with appropriate restriction enzyme, followed by gel analysis. Colonies checked to be plasmid bearing were put in 15 ml CG medium with ampicillin and incubated at 37 ℃ for 16 hr with shaking. The bacterial cells were harvested by spinning at 7,500 g for 5 min. and the plasmid DNA was collected by CONCERT$^{TM}$ Rapid Plasmid Miniprep System (GibcoBRL) according to the manufacturer's instructions.

### 2.5.5 Transient transfection of plasmid into SJD and ZFL cells

Cells ($2 \times 10^5$) were seeded in each well of 24-well culture plate. Construct containing 0.2 μg of 1.4kb zMT-II promoter gene (*zMT*-1.4-luc) and 0.2 μg of pRL-CMV (Promega) for normalization were mixed with 1 μl of Lipofactamine reagent (Invitrogen), 1μl of PLUS Reagent (Invitrogen) according to the manufacturer's instruction. The cells were transfected by adding the mixture into the wells and incubated for 3 hr. Promoter-less plasmid, pGL3-Basic (Promega) was also included in the transfection assay as negative control. The transfection reagents were removed form the cells after the 3 hr incubation and replaced with fresh culture medium with 5% serum, followed by incubation at 28 ℃ for 24 hr for recovery.

### 2.5.6 Heavy metal treatments and measurement of luciferase activities

The transfected cells treated with different heavy metal solutions at different dosages (0%, 10%, 25%, 50%, 75% and 100% 24h-$LC_{50}$ values) for 24 hr were harvested by the Passive Lysis Buffer (Promega) for luciferase activity measurement with the Dual-Luciferase® Reporter Assay System (Promega). The assay was carried out by using one fourth of reagent volume recommended in the technical manual. The firefly luciferase activity (zMT promoter) and *Renilla* luciferase (normalization) were measured by the Lumat LB 9501 luminometer. The firefly

luciferase activity was divided by their corresponding *Renilla* luciferase activity in order to determine the "relative luciferase activity", which was to normalize the experimental differences. The data was analysed by one-way ANOVA test performed by GrandPad Prism® analysis software on a personal computer.

# Chapter 3

# Results

## 3.1 Toxicities of various heavy metal ions

$EC_{50}$ values measured at 24 h for $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $As^{3+}$, $As^{5+}$, $Cr^{3+}$and $Cr^{6+}$ on the two cell lines were determined by alamarBlue™ assay, which is the assay that measuring the cellular proliferation. The result was calculated by using the GrandPad Prism® analysis software on a personal computer. Table 3.1 shows the 24h-$EC_{50}$ values of various metal ions for SJD and ZFL cell-lines. The values for $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, and $Hg^{2+}$ on SJD were obtained from Yan and Chan (2004) using the same alamerBlue™ assay method; all other values were determined in the present study. Fig. 3-1 shows the cyto-toxicity curves of various metals for SJD cell and Fig. 3-2 shows the cyto-toxicity curves of various metals for ZFL cell.

Generally speaking, SJD cell is sensitive to $Cd^{2+}$, $Hg^{2+}$ and $As^{3+}$, $Cr^{6+}$ also pose significant toxic effects on SJD. $Cu^{2+}$ and $Zn^{2+}$ pose intermediate toxic effects on SJD; whereas $Cr^{3+}$ and $As^{5+}$ could cause toxic effects on SJD at high concentrations. Similar pattern is observed in ZFL, except that $Cd^{2+}$ and $Cr^{6+}$ are less toxic to ZFL as compared with SJD. ZFL is also more sensitive to $As^{5+}$ than SJD, and less sensitive to $Cd^{2+}$ than SJD. Table 3-2 lists the cyto-toxic levels of various metal ions in rank

order from the most toxic metal ion to the less toxic ones.

Table3-1 The 24h-$EC_{50}$ values (μM) of various metal ions on ZFL and SJD cell lines

| Treatments | ZFL | SJD |
|---|---|---|
| $Zn^{2+}$ | 343.5 | 362 |
| $Cd^{2+}$ | 140.8 | 26.8 |
| $Cu^{2+}$ | 308 | 300 |
| $Hg^{2+}$ | 68.6 | 29.5 |
| $As^{3+}$ | 44.3 | 37.6 |
| $As^{5+}$ | 72.2 | 2007 |
| $Cr^{3+}$ | 3835 | 12039 |
| $Cr^{6+}$ | 440 | 133 |

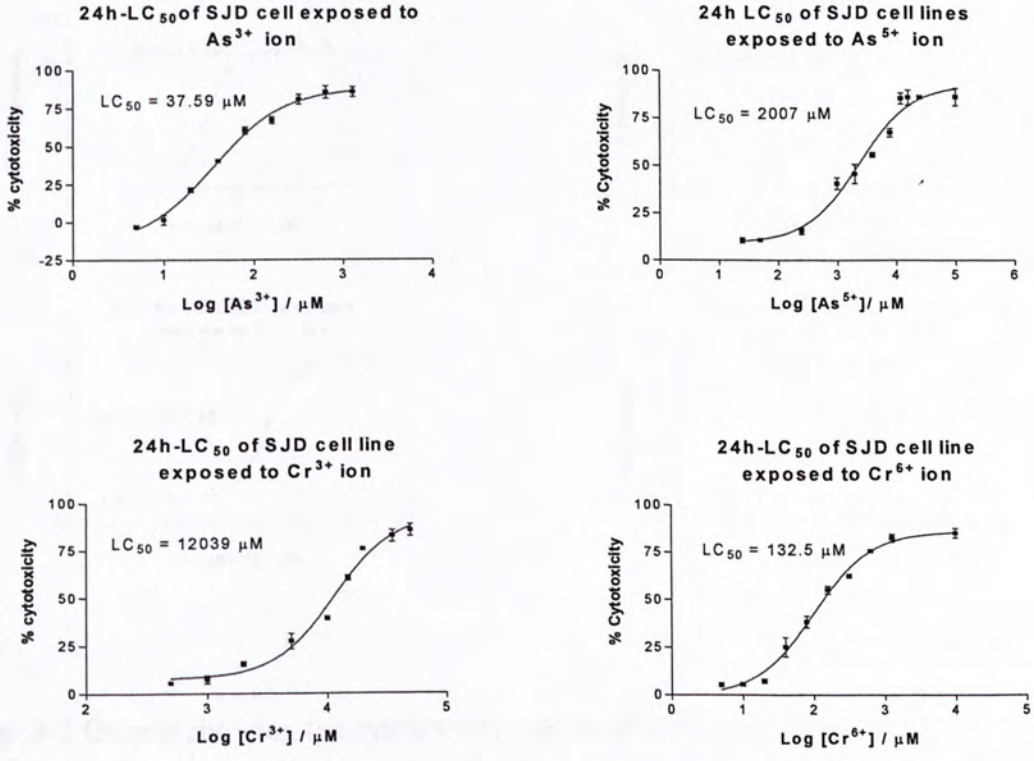



Figure 3-1 Graphs showing the cytotoxicity curves of $As^{3+}$, $As^{5+}$, $Cr^{3+}$and $Cr^{6+}$on the SJD cells after 24 hr exposure. Cytotoxicities were determined by the alamarBlue™

assays and presented as mean of 6 replicates ± S.D.

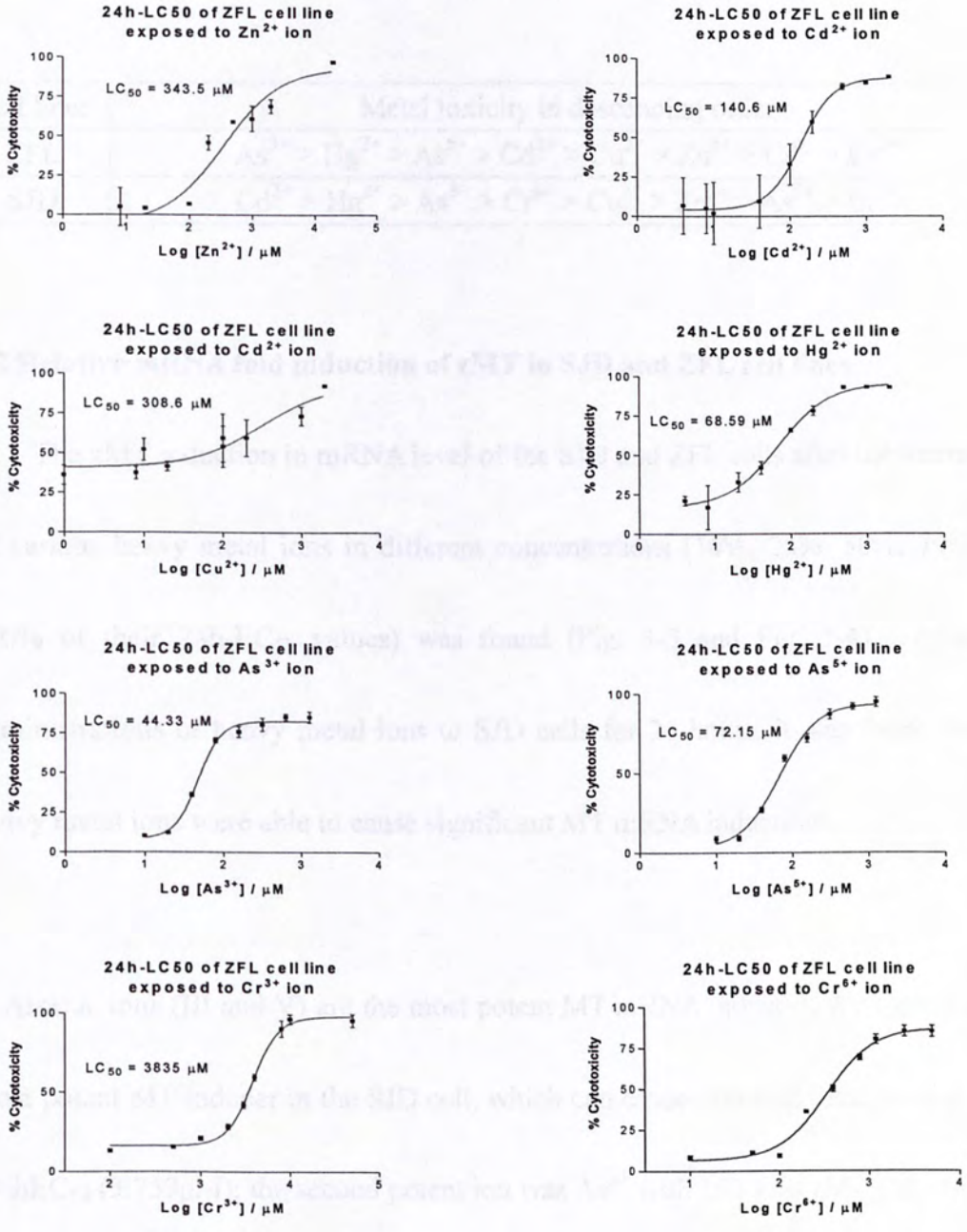



Fig. 3-2 Graphs showing the cytotoxicity curves of $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $As^{3+}$, $As^{5+}$, $Cr^{3+}$and $Cr^{6+}$ on the ZFL cells after 24 hr exposure. Cytotoxicities were determined by the alamarBlue™ assays and presented as mean of 6 replicates ± S.D.

Table 3-2 Rank orders of toxicities of different metal ions on ZFL and SJD cell lines obtained from the present studies

| Cell lines | Metal toxicity in descending order |
|---|---|
| ZFL | $As^{3+} > Hg^{2+} > As^{5+} > Cd^{2+} > Cu^{2+} > Zn^{2+} > Cr^{6+} > Cr^{3+}$ |
| SJD | $Cd^{2+} > Hg^{2+} > As^{3+} > Cr^{6+} > Cu^{2+} > Zn^{2+} > As^{5+} > Cr^{3+}$ |

### 3.2 Relative mRNA fold induction of zMT in SJD and ZFL cell lines

The zMT induction in mRNA level of the SJD and ZFL cells after the treatments of various heavy metal ions in different concentrations (10%, 25%, 50%, 75% and 100% of their 24h-$EC_{50}$ values) was found (Fig. 3-3 and Fig. 3-4). After the administrations of heavy metal ions to SJD cells for 24 hours, it was found that all heavy metal ions were able to cause significant MT mRNA inductions.

Arsenic ions (III and V) are the most potent MT mRNA inducers, $As^{5+}$ ion was the most potent MT inducer in the SJD cell, which can cause 660-fold inductions at 10% 24-h$EC_{50}$ (3.759μM); the second potent ion was $As^{3+}$ with 150-fold zMT inductions at 10% 24h-$EC_{50}$ (200.7μM); but both pose toxic effects on SJD cells leading to reduced MT mRNA fold induction with higher metal concentrations. Besides, $Cd^{2+}$ and $Zn^{2+}$ ions were able to cause over 200-fold and 120-fold inductions respectively at their 50% and 75% 24h-$EC_{50}$ ($Cd^{2+}$: 13.40 μM, $Zn^{2+}$: 271.4 μM) in SJD. Moderate

**Relative mRNA induction of MT in SJD cells**

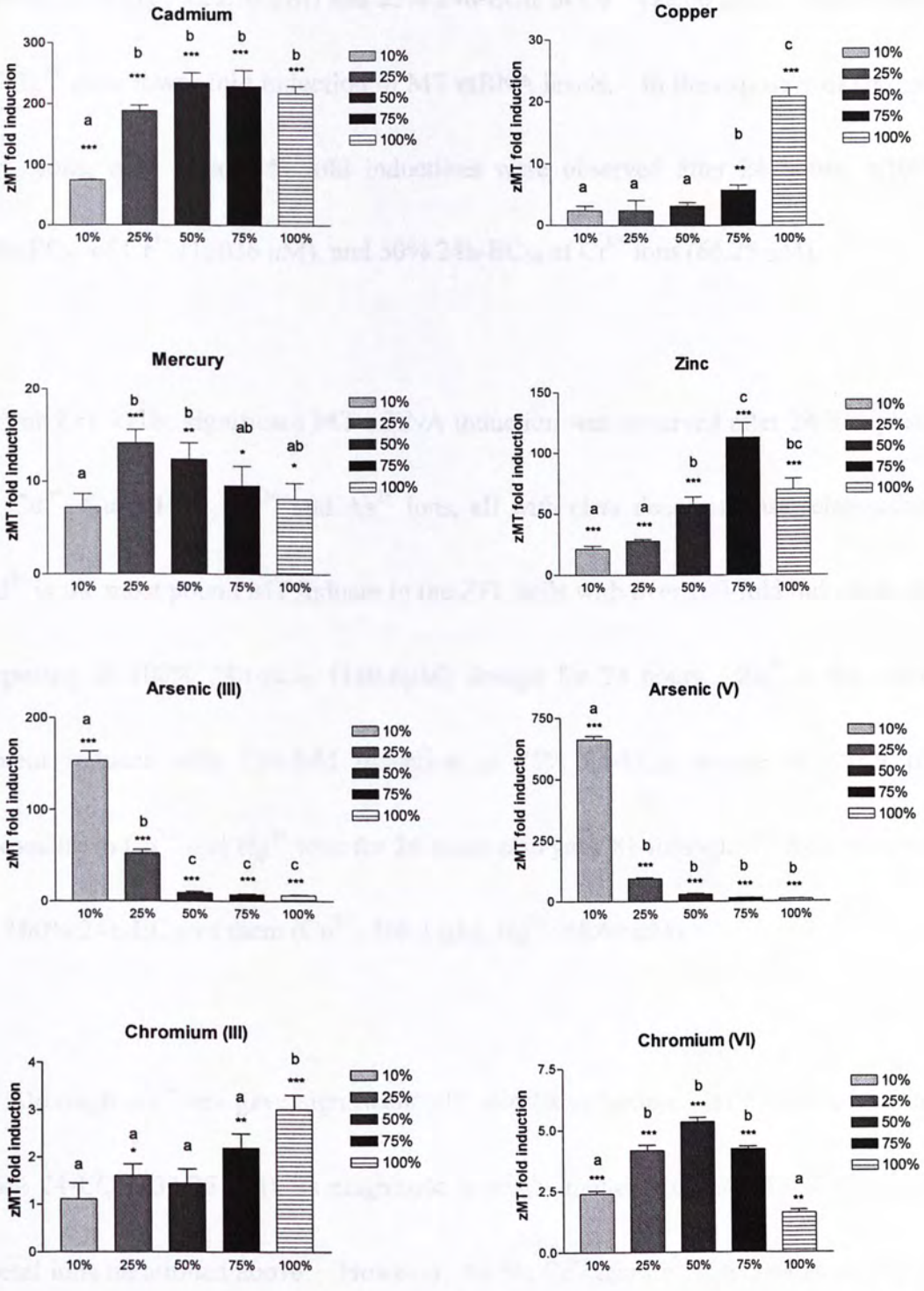



Fig 3-3 Graphs showing the relative MT fold inductions in SJD cells after 24h exposure to $Cd^{2+}$, $Cu^{2+}$, $Hg^{+}$, $Zn^{2+}$, $As^{3+}$, $As^{5+}$, $Cr^{3+}$and $Cr^{6+}$ at 10%, 25%, 50%, 75% and 100% of their 24h-$LC_{50}$ value. The results are represented as mean ± S.D. of 6 replicates. The data was analyzed by one-way ANOVA test.

inductions were observed in $Hg^{2+}$ (14-fold) and $Cu^{2+}$ (21-fold) exposure, at 100% 24h-$EC_{50}$ of $Hg^{2+}$ (29.50 μM) and 25% 24h-$EC_{50}$ of $Cu^{2+}$ (75.00 μM). Higher doses of $Hg^{2+}$ gave lower fold induction of MT mRNA levels. In the exposure of $Cr^{3+}$ and $Cr^{6+}$ ions, only 3- and 5- fold inductions were observed after 24 hours, at100% 24h-$EC_{50}$ of $Cr^{3+}$ (12036 μM), and 50% 24h-$EC_{50}$ of $Cr^{6+}$ ions (66.25 μM).

For ZFL cells, significant MT mRNA induction was observed after 24 h exposure to $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $Zn^{2+}$ and $As^{3+}$ ions, all with clear dose-response relationships. $Cd^{2+}$ is the most potent MT inducer in the ZFL cells with over 200-fold induction after exposing at 100% 24h-$EC_{50}$ (140.6μM) dosage for 24 hours. $Zn^{2+}$ is the second potent inducer with 130-fold induction at 75% 24-$EC_{50}$ dosage of 257.9 μM. Exposing in $Cu^{2+}$ and $Hg^{2+}$ ions for 24 hours also gave 81-fold and 52-fold inductions at 100% 24h-$EC_{50}$ of them ($Cu^{2+}$: 308.1 μM, $Hg^{2+}$: 68.60 μM).

Although $As^{3+}$ ion gave significant MT mRNA induction with 5-fold induction at 75% 24-$EC_{50}$ (33.25 μM), its magnitude is much smaller than that of the other heavy metal ions mentioned above. However, As 5+, $Cr^{3+}$ and $Cr^{6+}$ did not induce any MT mRNA in ZFL with all concentrations tested.

**Relative mRNA induction of zMT in ZFL cells**

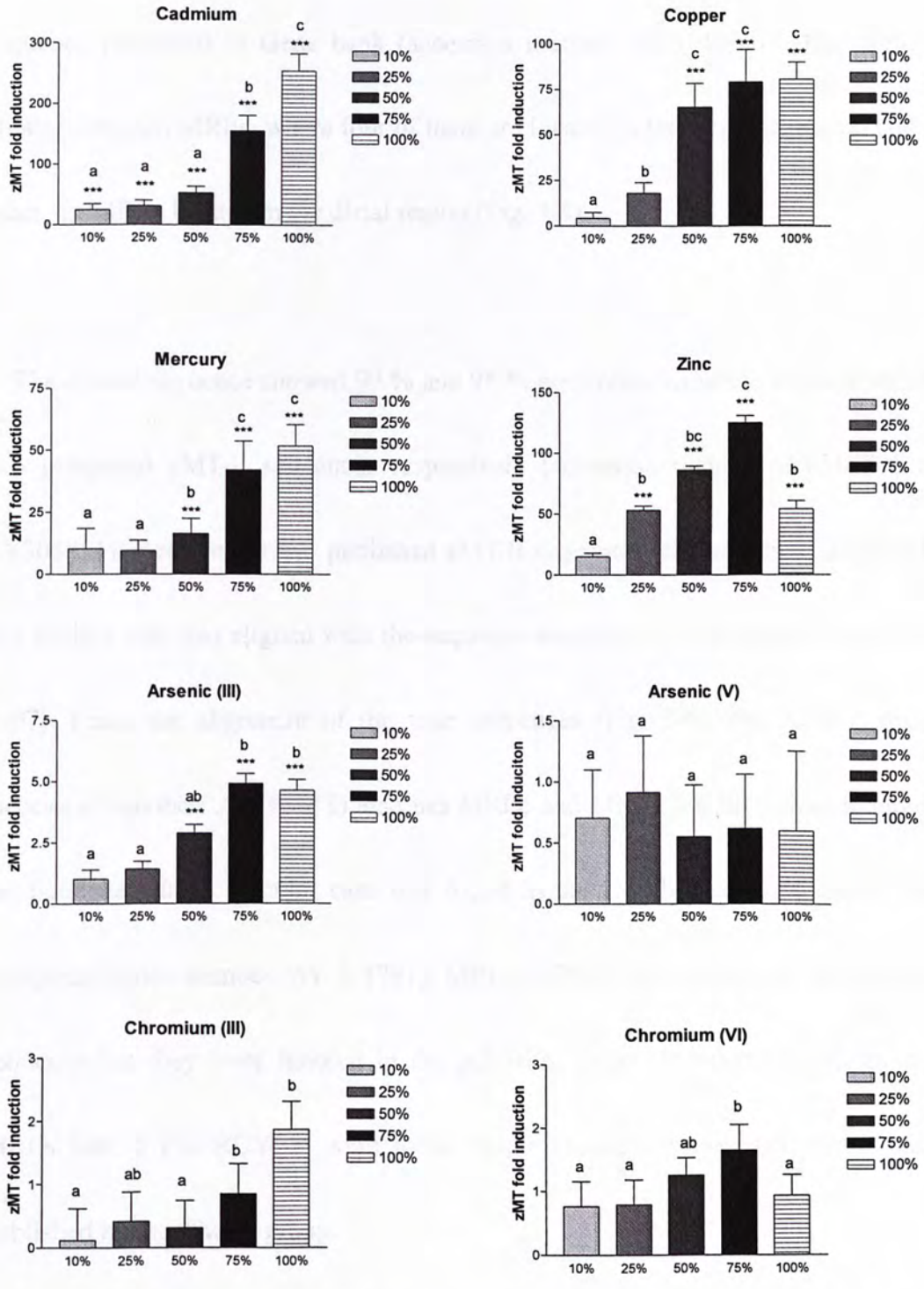



Fig 3-4 Graphs showing the relative MT fold inductions in ZFL cells after 24h exposure to $Cd^{2+}$, $Cu^{2+}$, $Hg^{+}$, $Zn^{2+}$, $As^{3+}$ and $As^{5+}$, $Cr^{3+}$and $Cr^{6+}$ at 10%, 25%, 50%, 75% and 100% of their 24h-$LC_{50}$ value. The results are represented as mean ± S.D. of 6 replicates. The data was analyzed by one-way ANOVA test.

### 3.3 The zMT-II gene and its induction by metal ions in zebrafish cell-lines

A 1.4kb zMT-II promoter was cloned by designing primers based on the sequence published in Gene bank (accession number: AY514791). The sequence obtained has ten MREs, where four of them are located in the proximal region and the other six MREs located in the distal region (Fig. 3-5).

The cloned sequence showed 99 % and 98 % nucleotide sequence identity with the two published zMT-II sequences respectively (accession number: AY514791 and AY305851). Besides the two published zMT-II sequences, the sequence amplified in this project was also aligned with the sequence amplified by Wan (unpublished data, 2007). From the alignment of the four sequences (Fig. 3-6), the 0.8kb sequence (accession number: AY305818) also has MREe and MREg but they were ignored in the published data. Similar case was found in the 1.4 kbp sequence from Chen's group (accession number: AY 514791), MREe, MREg and MREi were present in the sequence but they were ignored in the published data. In other words, ten exact MREs with 5'TGCRCYC3' were found in the cloned sequence and the sequence published by the Chen's group.

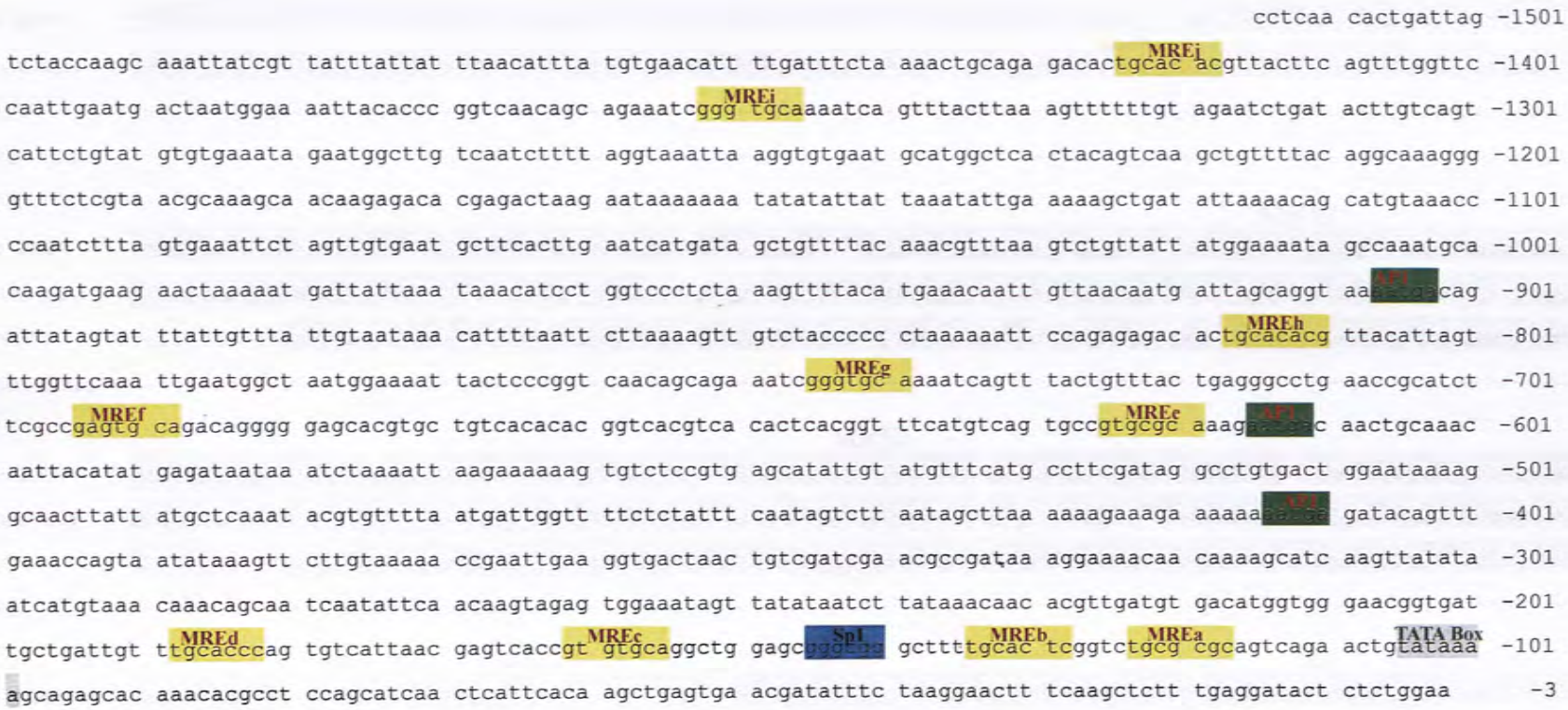



Fig. 3-5: Nucleotide sequence of the promoter region of zebrafish MT gene (zMT-II) used in this project. Ten putative MREs (5'TGCRCNC3")and a TATA box were identified in the promoter zMT-II. Putative AP1 site and Sp1 binding site are also shown.


Cheuk 2007 :---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----cctcaa cactgattag -1501
Chen et al. :---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----cctcaa cactgattag -1501
Wan 2007 :---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----cctcaa cactgattag -1501
Yan and Chan :---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -1501
MREj
Cheuk 2007 :tctaccaagc aaattatcgt tatttattat ttaacattta tgtgaacatt ttgatttcta aaactgcaga gacactgcac acgttacttc agtttggttc -1401
Chen et al. :tctaccaagc aaattatcgt tatttattat ttaacattca tgtgaacatt ttgatttcta aaactgcaga gacactgcac acgttacttc agtttggttc -1401
Wan 2007 :tctaccaagc aaattatcgt tatttattat ttaacattca tgtgaacatt ttgatttcta aaactgcaga gacactgcac acgttacttc agtttggttc
Yan and Chan :---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -1401
MREi
Cheuk 2007 :caattgaatg actaatggaa aattacaccc ggtcaacagc agaaatcggg tgcaaaatca gtttacttaa agtttttttgt agaatctgat acttgtcagt -1301
Chen et al. :caattgaatg actaatggaa aattacaccc ggtcaacagc agaaatcggg tgcaaaatta gtttacttaa agtttttttgc agaatctgat acttgtcagt -1301
Wan 2007 :caattgaatg actaatggaa aattacaccc ggtcaacagc agaaatcggg tgcaaaatta gtttacttaa agtttttttgc agaatctgat acttgtcagt -1301
Yan and Chan :---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -1301
Cheuk 2007 :cattctgtat gtgtgaaata gaatggcttg tcaatctttt aggtaaatta aggtgtgaat gcatggctca ctacagtcaa gctgttttac aggcaaaggg -1201
Chen et al. :cattctgtat gtgtgaaata gaatggcttg tgaatctttt tggtaaatta aggtgtgaat gcatggctca cttcagtcaa gctgttttac aggcaaaggg -1201
Wan 2007 :cattctgtat gtgtgaaata gaatggcttg tgaatctttt tggtaaatta aggtgtgaat gcatggctca cttcagtcaa gctgttttac aggcaaaggg
Yan and Chan :---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -1201

```
Cheuk 2007    :gtttctcgta acgcaaagca acaagagaca cgagactaag aataaaaaaa tatatattat taaatattga aaaagctgat attaaaacag catgtaaacc -1101
Chen et al.   :gtttctcgta acgcaaagca acaagagaca cgagactaag aataaaaaaa tatatattat taaatattga aaaagctgat attaaaacag catataaacc -1101
Wan 2007      :gtttctcgta acgcaaagca acaagagaca cgagactaag aataaaaaaa tatatattat taaatattga aaaagctgat attaaaacag catataaacc -1101
Yan and Chan  :---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -1101

Cheuk 2007    :ccaatcttta gtgaaattct agttgtgaat gcttcacttg aatcatgata gctgttttac aaacgtttaa gtctgttatt atggaaaata gccaaatgca -1001
Chen et al.   :ccaatcttta gtgaaattct agttgtgaat gcttcacttg aatcatgata gctgttttac aaacgtttaa gtctgttatt atggaaaata gccaaatgca -1001
Wan 2007      :ccaatcttta gtgaaattct agttgtgaat gcttcacttg aatcatgata gctgttttac aaacgtttaa gtctgttatt atggaaaata gccaaatgca -1001
Yan and Chan  :---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -1001

Cheuk 2007    :caagatgaag aactaaaaat gattattaaa taaacatcct ggtccctcta aagttttaca tgaaacaatt gttaacaatg attagcaggt aaaatgacag  -901
Chen et al.   :caagatgaag aactaaaaat gattattaaa taaacatcct ggtccctcta aagttttaca tgaaacaatt gttaacaatg attagcaggt aaaatgacag  -901
Wan 2007      :caagatgaag aactaaaaat gattattaaa taaacatcct ggtccctcta aagttttaca tgaaacaatt gttaacaatg attagcaggt aaaatgacag  -901
Yan and Chan  :---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  -901

                                                                                                                MREh
Cheuk 2007    :attatagtat ttattgttta ttgtaataaa cattttaatt cttaaaagtt gtctaccccc ctaaaaaatt ccagagagac actgcacacg ttacattagt  -801
Chen et al.   :attatagtat ttattgttta ttgtaatgaa cattttaatt cttaaaagtt gtctaccccc ctaaaaaatt ccagagagac actgcacacg ttacattagt  -801
Wan 2007      :attatagtat ttattgttta ttgtaatgaa cattttaatt cttaaaagtt gtctaccccc ctaaaaaatt ccagagagac actgcacacg ttacattagt  -801
Yan and Chan  :---------- ---------- ---------- -------------------- ---------- -----gaatt ccagagagac actgcacacg ttacattagt  -801
```


MREg
Cheuk 2007 :ttggttcaaa ttgaatggct aatggaaaat tactcccggt caacagcaga aatcgggtgc aaaatcagtt tactgtttac tgagggcctg aaccgcatct -701
Chen et al. :ttggttccaa ttgaatggct aatggaaaat tactcccggt caacagcaga aatcgggtgc aaaatcagtt tactgtttac tgagggcctg aaccgcatct -701
Wan 2007 :ttggttccaa ttgaatggct aatggaaaat tactcccggt caacagcaga aatcgggtgc aaaatcagtt tactgtttac tgagggcctg aaccgcatct
Yan and Chan :ttggttccaa ttgaatggct aatggaaaat tactcccggt caacagcaga aatcgggtgc aaaatcagtt tactgtttac tgagggcctg aaccgcatct -701
MREf
MREe
Cheuk 2007 :tcgccgagtg cagacagggg gagcacgtgc tgtcacacac ggtcacgtca cactcacggt ttcatgtcag tgccgtgcgc aaagaatgac aactgcaaac -601
Chen et al. :tcgccgagtg cagacagggg gagcacgtgc tgtcacacac ggtcacgtca cactcacagt ttcatgtcag tgccgtgcgc aaagaatgac aactgcaaac -601
Wan 2007 :tcgccgagtg cagacagggg gagcacgtgc tgtcacacac ggtcacgtca cactcacagt ttcatgtcag tgccgtgcgc aaagaatgac aactgcaaac -601
Yan and Chan :tcgccgagtg cagacagggg gagcacgtgc tgtcacacac ggtcacgtca cactcacagt ttcatgtcag tgccgtgcgc aaagaatgac aactgcaaac -601
Cheuk 2007 :aattacatat gagataataa atctaaaatt aagaaaaaag tgtctccgtg agcatattgt atgtttcatg ccttcgatag gcctgtgact ggaataaaag -501
Chen et al. :aattacatat gagataacaa atctaaaatt aagaaaaaag tgtctccgtg ggcatattgt atgtttcatg ccttcgatag gcatgtgact ggaataaaag -501
Wan 2007 :aattacatat gagataacaa atctaaaatt aagaaaaaag tgtctccgtg ggcatattgt atgtttcatg ccttcgatag gcatgtgact ggaataaaag -501
Yan and Chan :aattacatat gagataacaa atctaaaatt aagaaaaaag tgtctccgtg ggcatattgt atgtttcatg ccttcgatag gcatgtgact ggaataaaag -501
Cheuk 2007 :caacttatta tgctcaaata cgtgttttaa tgattggttt tctctatttc aatagtctta atagcttaaa aaaagaaaga aaaaaaatga gatacagttt -401
Chen et al. :caacttatta tgctcaaata cgtgttttaa tgtttgcttt tctctatttc aatagtctta atagcttaaa aaaagaaaga aaaaaaatga gatacagttt -401
Wan 2007 :caacttatta tgctcaaata cgtgttttaa tgtttgcttt tctctatttc aatagtctta atagcttaaa aaaagaaaga aaaaaaatga gatacagttt -401
Yan and Chan :caacttatta tgctcaaata cgtgttttaa tgtttgcttt tctctatttc aatagtctta atagcttaaa aaaagaaaga aaaaaaatga gatacagttt -401

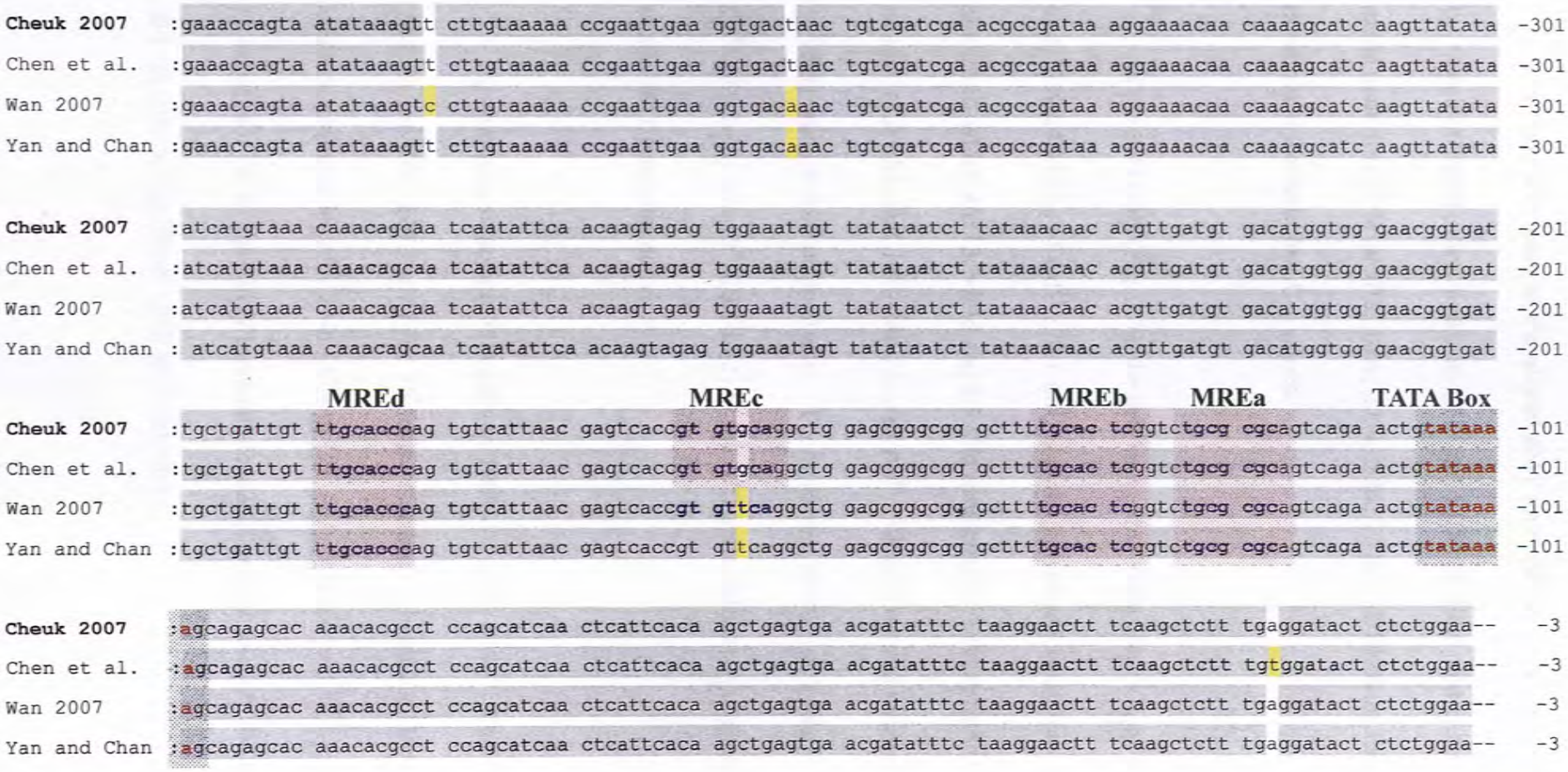



Fig. 3-6: Alignment of the nucleotide sequences obtained from Chen et al.., 2004 (accession number: AY 514791), Yan and Chan., 2004 (accession number: AY305818), sequence amplified by Wan (unplished data, 2007) and the sequence amplified in this project (Cheuk, 2007). The putative MREs (5'TGCRCNC3") and the TATA box are highlighted. The nucleotide difference in MREc may due to strain difference.

The responsiveness of the cloned zMT promoter to metal exposure was studied by transient transfection assay in ZFL and SJD cell lines and exposed to different metal ions for 24 hr at 0%, 10%, 25%, 50%, 75% and 100% of the concentrations determined for their 24-$LC_{50}$ values.

In the SJD cells (Fig. 3-7), the cell treated by 100% 24h-$LC_{50}$ of $Zn^{2+}$ (361.8 μM) and 75% 24h-$LC_{50}$ of $Cd^{2+}$ (20.1 μM) showed significant inductions of transcription activities, which are 13.1-fold and 6.5 fold respectively. Treatment of 10% 24h-$LC_{50}$ of $Hg^{+}$ (3.0 μM) and 10% 24h-$LC_{50}$ of $As^{3+}$ (3.8 μM) caused moderate induction of 3.2-fold and 1.9-fold respectively. The treatment of $Cu^{2+}$ (1.1-fold), $As^{5+}$ (1.4-fold), $Cr^{3+}$ (0.9-fold) and $Cr^{6+}$ (0.8-fold) to the SJD cells did not give significant induction of the zMT promoter activity.

In summary, exposing SJD cells in 100% 24-$LC_{50}$ of $Zn^{2+}$ (361.8 μM) and 75% 24-$LC_{50}$ of $Cd^{2+}$ (20.1 μM) for 24 hr gave strong induction of the zMT promoter, whereas exposed in 10% 24-h $LC_{50}$ of $Hg^{+}$ (3.0 μM) ion and 10% 24h-$LC_{50}$ of $As^{3+}$ (3.8 μM) for 24 hr gave only moderate levels of gene activation and other metal ions did not induce significant zMT gene activities.

For ZFL cells (Fig. 3-8), similar result was found. Exposure to 100% 24h-$LC_{50}$ of zinc and 10% 24-h $LC_{50}$ of cadmium ions caused significant induction of the zMT promoter transcription activity, which are 6.8-fold and 4.8-fold respectively. For the mercury ion, which caused moderate induction in the SJD cell, was not able to cause significant induction in the ZFL cell (1.7-fold). However, the exposure of 10% 24h-$LC_{50}$ of arsenic (III) ion could cause statistically significant induction (1.7-fold) in ZFL cells. Same to the result of SJD cells, the treatment of copper (1.0-fold), arsenic (V) (1.1-fold), chromium (III) (1.0-fold) and chromium (VI) ions (0.5-fold) to the ZFL cells could not cause significant induction in the zMT promoter activity.

To conclude, both cell lines showed sensitive and significant fold induction of the zMT gene promoter following the administrations of $Cd^{2+}$ and $Zn^{2+}$, while $As^{3+}$ are the moderate inducer to the two cell lines. For the other heavy metal ions, only $Hg^{2+}$ gave moderate but weak induction in the SJD cells. SJD cells showed greater sensitivities and responsiveness of zMT gene promoter corresponding to the heavy metal exposure than ZFL cells.

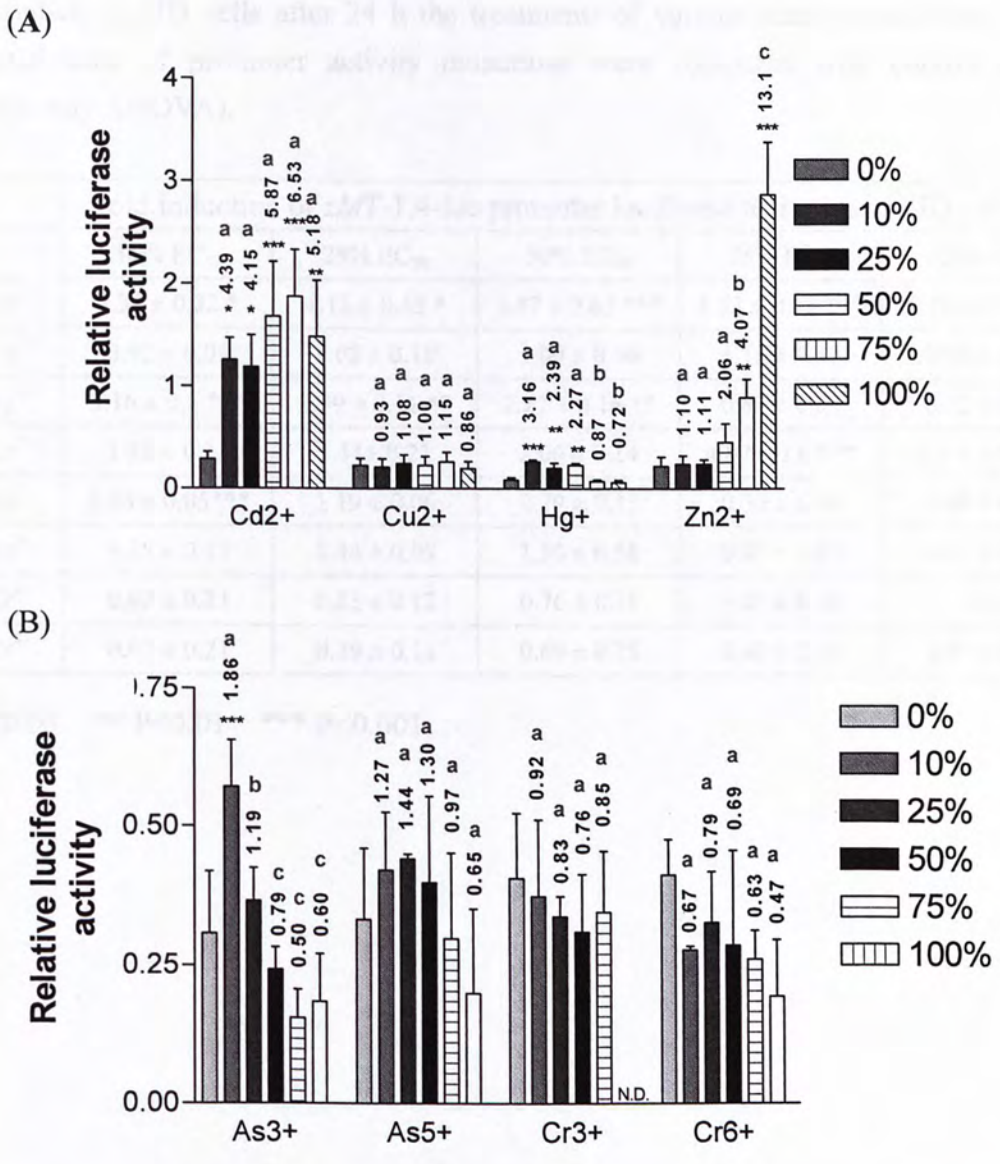



Fig. 3-7 Transcription levels of *zMT*-1.4-Luc transfected in SJD cells following the administrations of various metal ions. Transfected cells were treated with (A) $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Hg^{+}$ and (B) $As^{3+}$, $As^{5+}$, $Cr^{3+}$ and $Cr^{6+}$ in the dosages corresponding to 0% 25%, 50%, 75% and 100% of their LC50 values for 24 h. Each value represents the mean of ± S.D. of three replicates. The data was analyzed by one-way ANOVA test.

Table 3-3: Fold inductions of *zMT*-1.4-luc promoter levels as measured for luciferase activities in SJD cells after 24 h the treatments of various heavy metal ions. The significance of promoter activity inductions were compared with control group (One-way ANOVA).

| | Fold induction of *zMT*-1.4-luc promoter luciferase activities in SJD cell line | | | | |
|---|---|---|---|---|---|
| | 10% $EC_{50}$ | 25% $EC_{50}$ | 50% $EC_{50}$ | 75% $EC_{50}$ | 100% $EC_{50}$ |
| $Cd^{2+}$ | 4.39 ± 0.32 * | 4.15 ± 0.55 * | 5.87 ± 0.63 *** | 6.53 ± 0.19 *** | 5.18 ± 1.71 ** |
| $Cu^{2+}$ | 0.93 ± 0.09 | 1.08 ± 0.10 | 1.00 ± 0.16 | 1.15 ± 0.41 | 0.86 ± 0.09 |
| $Hg^{2+}$ | 3.16 ± 0.1 *** | 2.39 ± 0.11 ** | 2.27 ± 0.16 ** | 0.87 ± 0.09 | 0.72 ± 0.06 |
| $Zn^{2+}$ | 1.10 ± 0.22 | 1.11± 0.21 | 2.06 ± 0.14 | 4.07 ± 1.07 ** | 13.1 ± 1.94 *** |
| $As^{3+}$ | 1.86 ± 0.05*** | 1.19 ± 0.06 | 0.79 ± 0.15 | 0.50 ± 0.06 | 0.60 ± 0.07 |
| $As^{5+}$ | 1.27 ± 0.13 | 1.44 ± 0.09 | 1.30 ± 0.56 | 0.97 ± 0.33 | 0.65 ± 0.20 |
| $Cr^{3+}$ | 0.92 ± 0.21 | 0.83 ± 0.12 | 0.76 ± 0.11 | 0.85 ± 0.16 | N.D. |
| $Cr^{6+}$ | 0.67 ± 0.21 | 0.79 ± 0.11 | 0.69 ± 0.25 | 0.63 ± 0.13 | 0.47 ± 0.12 |

$P<0.05$ ** $P<0.01$ *** $P<0.001$

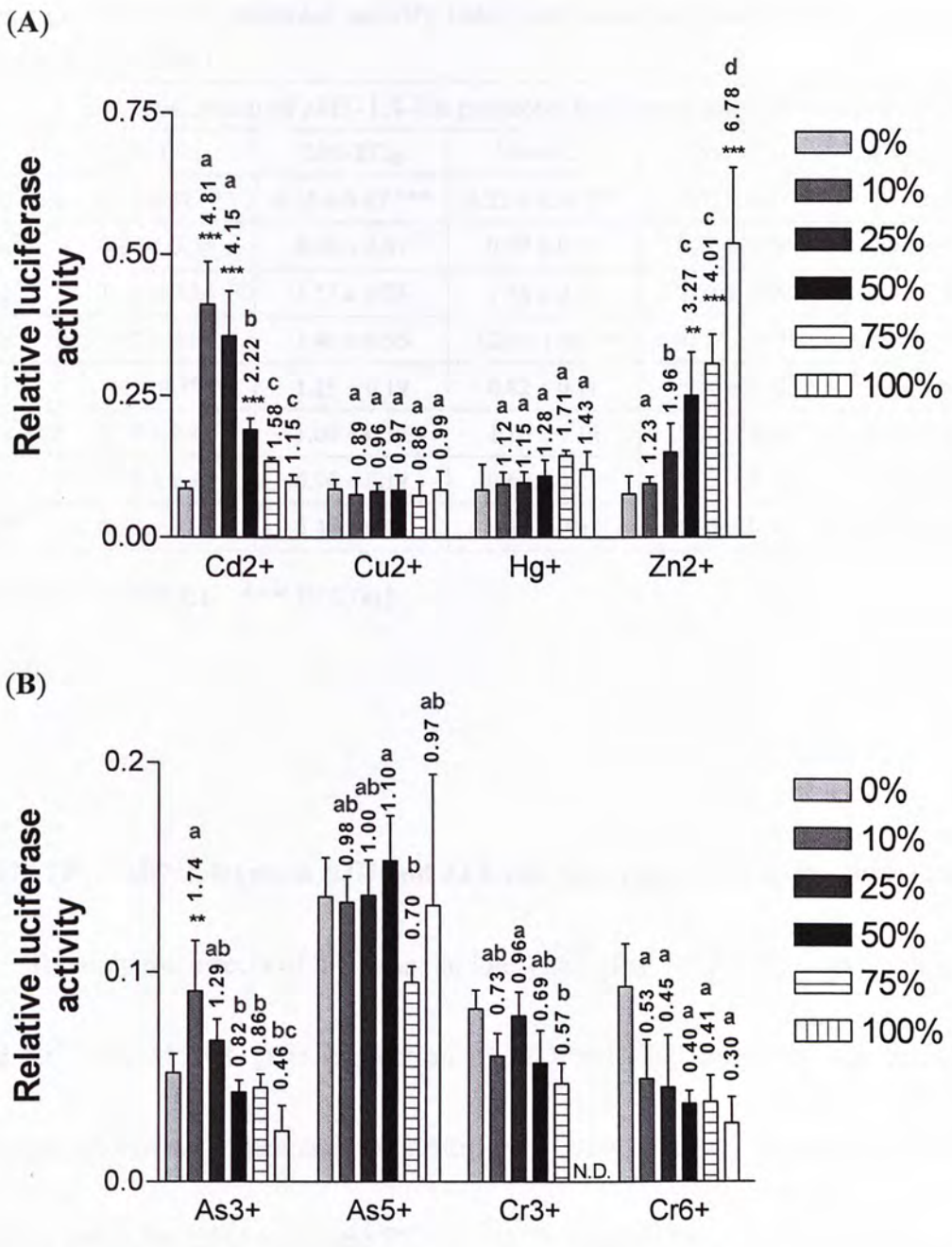



Figure 3-8 Transcription levels of *zMT*-1.4-Luc transfected in ZFL cells following the administrations of various metal ions. Transfected cells were treated with (A) $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Hg^{+}$ and (B) $As^{3+}$, $As^{5+}$, $Cr^{3+}$ and $Cr^{6+}$ in the dosages corresponding to 0% 25%, 50%, 75% and 100% of their LC50 values for 24 h. Each value represents the mean of ± S.D. of three replicates. The data was analyzed by one-way ANOVA test.

Table 3-4: Fold inductions of *zMT*-1.4-luc promoter as represented by measurements of luciferase activities in ZFL cells after 24 h treatments of various heavy metal ions. The significance of promoter activity inductions were compared with control group (One-way ANOVA).

| | Fold induction of *zMT*-1.4-luc promoter luciferase activities in ZFL cell line | | | | |
|---|---|---|---|---|---|
| | 10% $EC_{50}$ | 25% $EC_{50}$ | 50% $EC_{50}$ | 75% $EC_{50}$ | 100% $EC_{50}$ |
| $Cd^{2+}$ | 4.81 ± 0.31 *** | 4.15 ± 0.47 *** | 2.22 ± 0.20 *** | 1.58 ± 0.17 | 1.15 ± 0.02 |
| $Cu^{2+}$ | 0.89 ± 0.35 | 0.96 ± 0.17 | 0.97 ± 0.32 | 0.86 ± 0.30 | 0.99 ± 0.38 |
| $Hg^{2+}$ | 1.12 ± 0.24 | 1.15 ± 0.23 | 1.28 ± 0.27 | 1.71 ± 0.09 | 1.43 ± 0.36 |
| $Zn^{2+}$ | 1.23 ± 0.08 | 1.96 ± 0.55 | 3.27 ± 1.09 ** | 4.01 ± 0.37 *** | 6.78 ± 1.75 *** |
| $As^{3+}$ | 1.74 ± 0.39** | 1.29 ± 0.19 | 0.82 ± 0.11 | 0.86 ± 0.12 | 0.46 ± 0.22 |
| $As^{5+}$ | 0.98 ± 0.12 | 1.00 ± 0.13 | 1.10 ± 0.16 | 0.70 ± 0.06 | 0.97 ± 0.44 |
| $Cr^{3+}$ | 0.73 ± 0.12 | 0.96 ± 0.14 | 0.69 ± 0.21 | 0.57 ± 0.13 | N.D. |
| $Cr^{6+}$ | 0.53 ± 0.21 | 0.45 ± 0.24 | 0.40 ± 0.07 | 0.41 ± 0.14 | 0.30 ± 0.13 |

$P<0.05$ ** $P<0.01$ *** $P<0.001$

### 3.4 MTF-1 mRNA levels in SJD and ZFL cell lines exposed to heavy metal ions

To study the effects of heavy metal ions ($Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Hg^{+}$, $As^{3+}$, $As^{5+}$, $Cr^{3+}$ and $Cr^{6+}$) on MTF-1 gene expression in SJD and ZFL cell lines, the cells were exposed to various concentrations of different metal ions and measured for MTF-1 mRNA levels by using real-time PCR. Fig. 3-9 shows the results obtained for SJD cell line, significant MTF-1 mRNA inductions were observed in $Cd^{2+}$, $As^{3+}$, $As^{5+}$, $Cr^{3+}$ and $Cr^{6+}$ exposure for 12 h. Among these heavy metal ions, $Cd^{2+}$ exposure caused the greatest MTF-1 induction, which is 11-fold at 75% 24h-$EC_{50}$ dosage (20.1 μM). $As^{3+}$ and $As^{5+}$, exposure caused 8.6-fold and 5.4-fold induction at 25% 24h-$EC_{50}$ (9.4

μM) and 50% 24h-$EC_{50}$ dosage (1004 μM) respectively. 3.4-fold MTF-1 mRNA induction was observed in $Cr^{6+}$ exposure at 50% 24h-$EC_{40}$ dosage (66.3 μM) while the induction caused by $Cr^{3+}$ exposure was low (2-fold) at 50% 24h-$EC_{50}$ (6020 μM). For the cells exposed in $Zn^{2+}$, $Cu^{2+}$ and $Hg^{+}$, no significant MTF-1 induction in mRNA level was observed.

For ZFL cell line (Fig. 3-10), $Cd^{2+}$ was also the greatest inducer of MTF-1 in mRNA level. 9.4-fold induction was observed at 25% 24h-$EC_{50}$ (35.2 μM). $Hg^{+}$ gave no significant MTF-1 induction caused in the SJD cell line, but it gave 5.4-fold induction ZFL cells exposure at 50% 24h-$EC_{50}$ (34.3 μl).

$As^{3+}$, $As^{5+}$, and $Cr^{6+}$ exposures were able to induce MTF-1 mRNA level in SJD cell, but they did not give significant MTF-1 induction in ZFL cell line. Similar to the SJD cell line, exposure in $Zn^{2+}$, $Cu^{2+}$ and $Cr^{3+}$ were not able to cause significant induction of MTF-1 mRNA levels in ZFL cell neither.

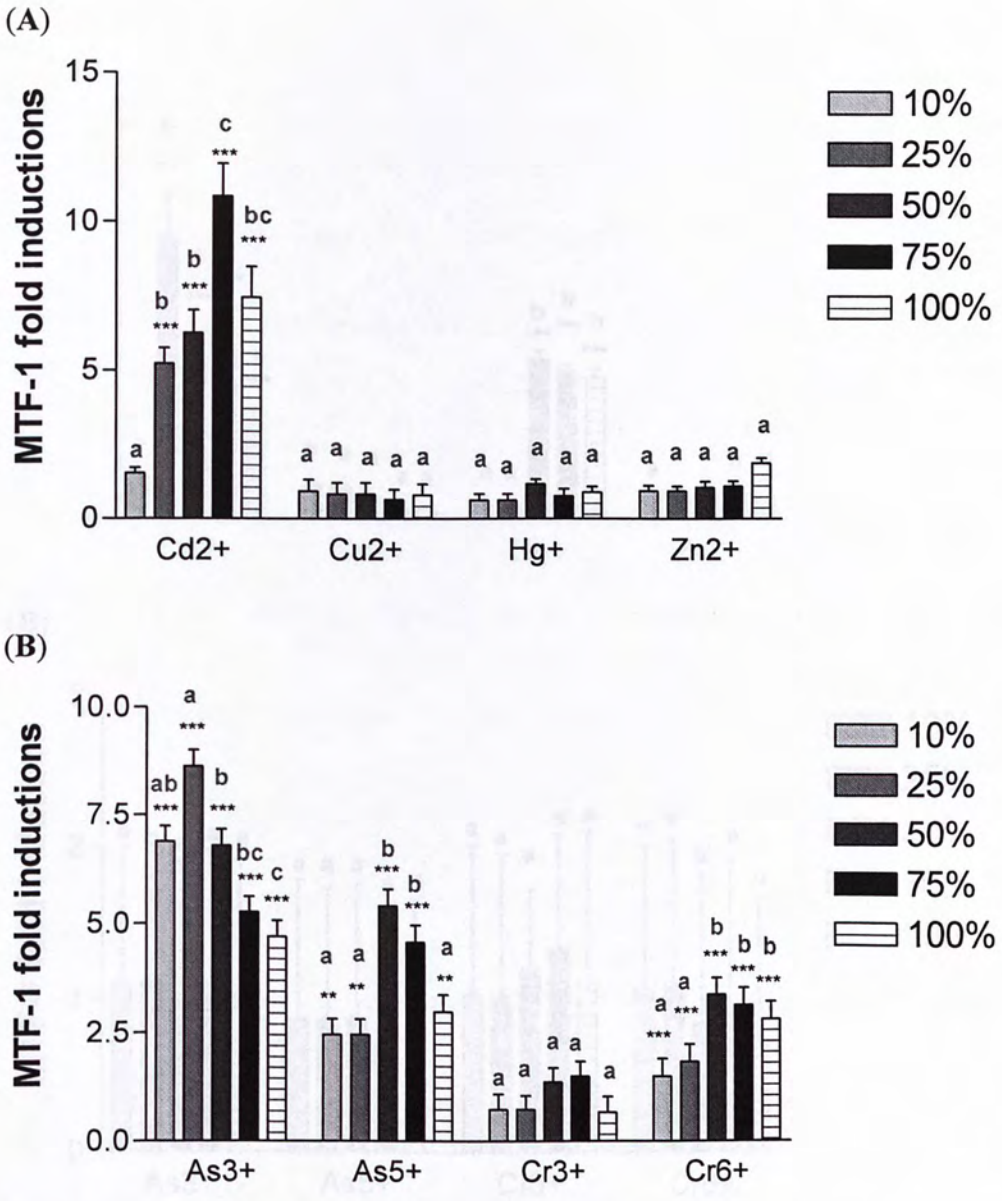



Fig. 3-9 Relative fold inductions of MTF-1 mRNA levels over control in SJD cells after 12h exposure to $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Zn^{2+}$ at 10%, 25%, 50%, 75% and 100% of their 24h-$LC_{50}$ value (A) and $As^{3+}$, $As^{5+}$, $Cr^{3+}$ and $Cr^{6+}$ at 10%, 25%, 50%, 75% and 100% of their 24h-$LC_{50}$ value (B). The results are represented as mean ± S.D. of 6 replicates. The data was analyzed by one-way ANOVA test.

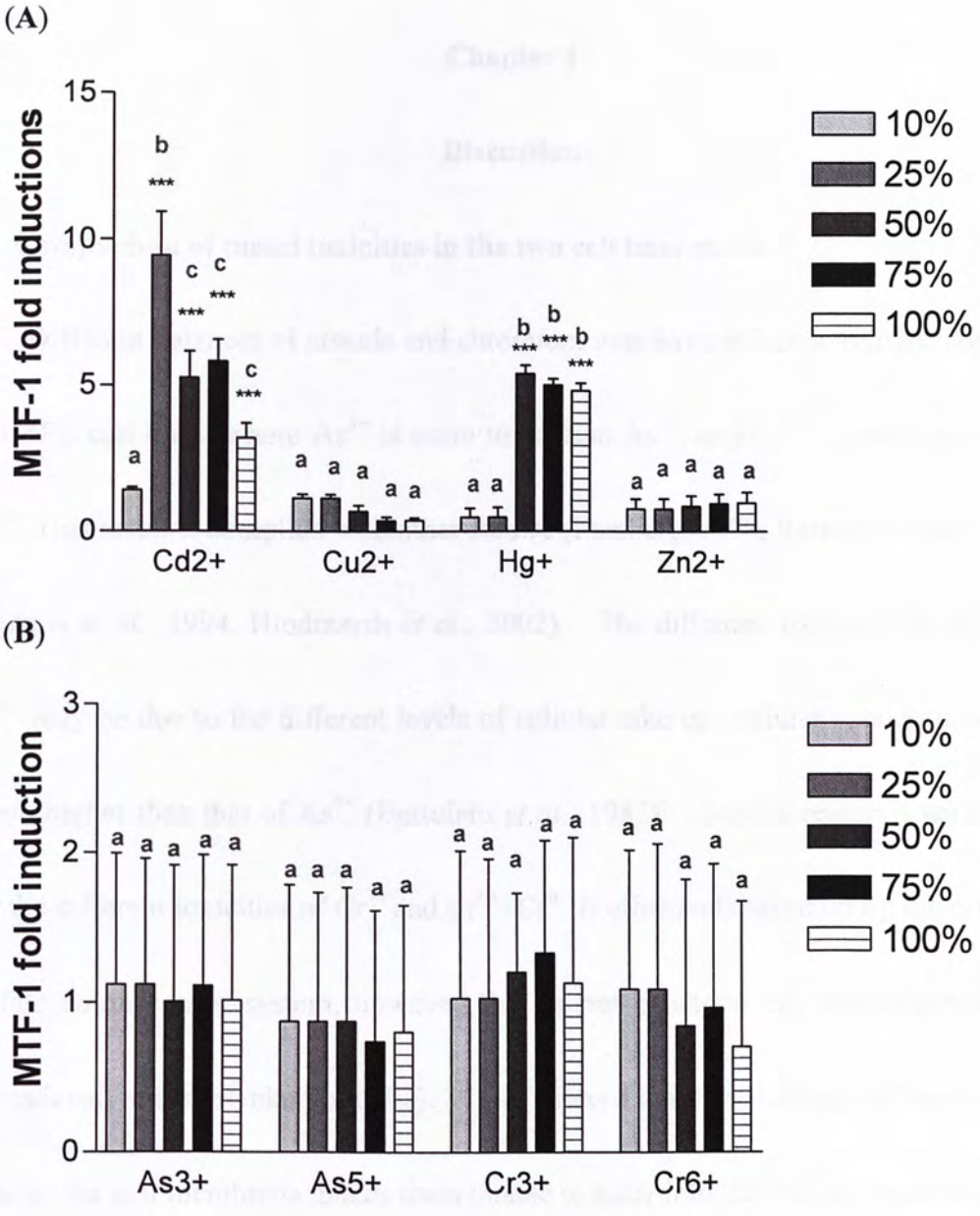



Fig. 3-10 Relative fold inductions of MTF-1 mRNA level in ZFL cells after 12h exposure to $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Zn^{2+}$ at 10%, 25%, 50%, 75% and 100% of their 24h-$LC_{50}$ value (A) and $As^{3+}$, $As^{5+}$, $Cr^{3+}$ and $Cr^{6+}$ at 10%, 25%, 50%, 75% and 100% of their 24h-$LC_{50}$ values (B). The results are represented as mean ± S.D. of 6 replicates. The data was analyzed by one-way ANOVA test.

# Chapter 4

# Discussion

## 4.1 Comparison of metal toxicities in the two cell lines studied

Different valances of arsenic and chromium ions have different toxicities on SJD and ZFL cell lines, where $As^{3+}$ is more toxic than $As^{5+}$, and $Cr^{6+}$ is more toxic than $Cr^{3+}$. This result is complied with other studies (Forsberg, 1978, Bertolero *et al.,* 1987, Ramana *et al.*, 1994, Hindmarsh *et al.*, 2002). The different toxicities of $As^{3+}$ and $As^{5+}$ may be due to the different levels of cellular take up, cellular uptake of $As^{3+}$ is much higher than that of $As^{5+}$ (Bertolero *et al.*, 1987). Similar reason is accounted for the different toxicities of $Cr^{3+}$ and $Cr^{6+}$: $Cr^{6+}$ is effectively taken up by the cells via sulfate anion carrier system, however $Cr^{3+}$ cannot penetrate cell membranes easily (Casadevall and Kortenkamp, 2002). It is believed that the blockage of heavy metal ions by the cell membrane makes them unable to enter into the cell and thus unable to exert their toxic effect.

Comparing the results obtained from the two cell lines, it was found that the $EC_{50}$ values of the heavy metal ions to the two cell lines are very different. For example, the $EC_{50}$ value of $Cd^{2+}$ to ZFL cells is 140.6 μM, while it is 26.8 μM to SJD cells, indicated that ZFL cells is slightly more tolerant to $Cd^{2+}$ than SJD cells. On the

other hand, $EC_{50}$ value of $As^{5+}$ to ZFL cells is 72.15 μM, but it is 2007 μM to SJD cells, SJD cells is more tolerant to $As^{5+}$ than ZFL cells. This result shows that different organs have different tolerance to the heavy metal ions.

ZFL is the cells from the zebrafish liver, which is an organ for the metabolism, many xenobiotics or toxic substances, are metabolite there. The liver converts the toxic substances into less toxic or even non-toxic one, this is known as detoxifying mechanism. On the other hand, SJD cells are the fin cells of zebrafish, which is mainly responsible for the movement of the fish. Thus the main metabolism in there may just for respiration, protection from external invasion, it is believed that SJD cells contain less enzyme for detoxification as compared with the liver cells. Therefore the presence of detoxifying system can be one of the reasons that ZFL cells have higher tolerance to several metal ions than SJD cells.

However SJD cells show higher tolerance in some heavy metal ion exposures, this is due to many mechanisms of the enzymes is still unknown and detoxification is just one possible mechanism of them. Moreover, the presence of receptors or membrane transporters also affects the toxicity of metal ions on cells as all metal ions must enter the cell through the membranes. Therefore, less receptor or transporters

present in SJD cell is another possibility for reduced toxic effects. As the mechanisms of the heavy metal ions on the two cell line are unknown, a clear conclusion cannot be drawn unless further information on metal transporters is available.

## 4.2 zMT gene expression study

### 4.2.1 zMT mRNA regulation by heavy metal ions in the two cell lines

Among the heavy metal ions, $Zn^{2+}$ and $Cd^{2+}$ ions are both potent zMT inducers in ZFL cells and SJD cells. These two heavy metal ions were well known MT inducers, therefore it is not surprised that they gave significant zMT mRNA inductions in the two cell lines. However, $Cd^{2+}$ gave greater induction (~ 250-folds) in the two cell lines when compare with $Zn^{2+}$ (~120-folds), even $Zn^{2+}$ is regarded as the primary inducer of MT. This can be due to the metal homeostasis mechanisms other than MT regulation presence in the zinc ion regulation, such asGSH conjugation and ZnT-1-mediated regulation (Langmade *et al.*, 2000), can share the burden of MT. $Cu^{2+}$ and $Hg^{2+}$ also gave significant inductions in the two cell lines, but the magnitude of fold inductions (1 – 3) are much lower than that of zinc and cadmium ions.

The MT mRNA responses were different in the two cell lines when exposing to

$As^{3+}$, $As^{5+}$, $Cr^{3+}$and $Cr^{6+}$. The MT mRNA responses to $As^{3+}$ and $As^{5+}$ in ZFL were weak or even no response observed in many doses, however the MT mRNA inductions in SJD caused by $As^{3+}$ and $As^{5+}$ at 10% 24h-$EC_{50}$ dosages were very high that they were comparable with the inductions caused by $Zn^{2+}$ and $Cd^{2+}$. Although the MT mRNA inductions caused by $As^{3+}$ and $As^{5+}$ exposures in SJD cells were decreased with higher doses, the fold inductions caused in high dosages were still significant from statistical analysis.

For the chromium ion exposure, both $Cr^{3+}$and $Cr^{6+}$ were not able to cause significant zMT induction in ZFL cells. However they were regarded as weak inducer in the SJD cells, which could cause ~3 and ~5 fold of MT mRNA inductions respectively.

After comparing the inductions in the two cell lines caused by the various heavy metal ions, it was found that all of the eight heavy metal ions investigated did cause significant MT mRNA inductions in SJD cells. But for ZFL cells, only $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $As^{3+}$ gave significant inductions. To conclude, SJD is responsive to more heavy metal ions in MT gene expression in mRNA level and so it would be a better model in studying the MT gene expression for bio-monitoring purposes.

### 4.2.2 The potential use of MT regulation as exposure biomarker

This project studied the regulation of MT gene in transcriptional level mediated by $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $As^{3+}$, $As^{5+}$, $Cr^{3+}$and $Cr^{6+}$ in the ZFL and SJD cell lines by using the real-time PCR technique. We used real-time PCR with the high sensitivity and reliability of using SYBR green based staining technique in the detection of the target gene (zMT) and this method is relatively rapid, convenient, and can study a large number of samples in one time. Through the use of this technique, the MT mRNA inductions of different heavy metal ions were demonstrated in the two cell lines and it was found that $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $As^{3+}$ exposures in the two cell lines can induce the MT expression. Therefore, the use of these two zebrafish cell line with the zMT mRNA level as exposure marker can be a sensitive and feasible method to determine the bioavailability of these ions to the aquatic organisms.

## 4.3 Structure of the zMT-II gene promoter region

In vertebrates, a typical MRE consensus sequence is present in all MT promoters with multiple copies and they are important components in the mediation of response to metals (Varshney *et al.*, 1986, Searle *et al.,* 1987, Culotta and Hamer, 1989, Scudiero *et al.,* 2001). Although the MRE sequence is highly conserved, the number of MRE present in the promoter region is varied among different species. For

example, seven MREs were present in the MT-II promoter region of icefish and tilapia MT-I promoter consists of six MREs (Scudiero *et al.*, 2001; Chan, 2007). For rainbow trout, six MREs are located in the MT-A promoter region and two MREs are present in the MT-B promoter region (Olsson *et al.*, 1995; Samson and Gedamu, 1995).

From this study, the promoter in zebrafish contains ten MREs. Four of them (MREa to MREd) were distributed in the proximal region within 100 bp from the TATA box. The first MRE (MREa, TGCGCGC) and the second MRE (MREb, TGCACTC) were located from -118 to -124 and -129 to -135 upstream of the transcription starting point respectively. The third one (MREc, TGCACAC) was reverse oriented and located from -156 to -162 and the fourth one (MREd, TGCACCC) was located between –183 and -189. Another six were organized in the distal cluster, MREe (TGCGCAC), MREf (TGCACTC) and MREg (TGCACCC) was reverse oriented and located from -620 to -626, -689 to -695 and -740 to -746 respectively. MREh (TGCACAC) was located form -812 to -818, MREi (TGCACCC) was reverse oriented from -1347 to -1353 and the last one MREj (TGCACAC) was located form -1419 to -1425.

## 4.4 Metal responsiveness of zMT-II promoter

The metal responsiveness of zMT-II promoter were studies by exposing the SJD and ZFL cells that transfected with the zMT-II promoter into different heavy metals for 24 h, the responsiveness of the zMT-II promoter were determined by the luciferase assay. $Zn^{2+}$ and $Cd^{2+}$ were found able to elicits MT gene expression at the promoter level in many studies (Olsson *et al.,* 1995, Andrews,1990 Andrews, 2000, Bourdineaud *et al.,* 2006). Similar result was obtained in the SJD and ZFL cells, the results of the two cell lines indicated that $Zn^{2+}$ was the most potent MT inducer in the promoter level, followed by $Cd^{2+}$, which can cause 13-fold and 6.5- fold inductions in SJD cell and 6.8- fold and 4.8-fold to ZFL cells respectively.

Although MT is believed to be one of the mechanisms in avoiding the toxicity effects caused by the excess $Cu^{2+}$ (Mehta *et al.,* 2006), no significant MT inductions were observed in the ZFL (1.1-fold) and SJD cells (1.0-fold) in the promoter level after the administrations of $Cu^{2+}$. Similar result was obtained in the study of rainbow trout MT-A gene that both $Zn^{2+}$ and $Cd^{2+}$ were found to be good inducers and $Cu^{2+}$ was a poor inducer (Olsson *el al.,* 1995). The low ability in MT promoter activation of $Cu^{2+}$ may be due to its inability to directly activate metal transcription through MRE, it was proposed that $Cu^{2+}$ may induce MT through $Zn^{2+}$ pool adjustment that is

displaced from high affinity sites in other marcomolecules (Palmiter *et al.,* 1994, Olsson *et al.,* 1995).

Comparing the zMT induction in mRNA and promoter level (Table 4-1), the responsiveness of the two cell lines to the heavy metal ions was higher in mRNA level. For $As^{3+}$and $As^{5+}$ exposure in SJD cells, the MT mRNA induction were 250-fold and 600-fold respectively, however only 1.9-fold induction was observed in the promoter level of $As^{3+}$ exposure and there was no response for $As^{5+}$ exposure. The difference of the result is due to the two assays used in the two studies are different. The real-time PCR assay is measuring the production of MT mRNA while luciferase assay is measuring the enzyme activity and the higher sensitivity of the real-time PCR assay may account for the different result of the two assays. Besides, promoter study was studying the response of zMT-II promoter, however the study in mRNA level was studying the all MT mRNA. As mentioned in the introduction, zebrafish has two MT isoforms, MT-A and MT-II. Previous study proved that the two MT isoforms response differently with the heavy metal treatment and the induction extend of the two isoforms were also different in different organs (Gonzalez *et al.,* 2006). In other words, the zMT isoforms might have specificity to different heavy metal ions as well as different organs. Due to the presence of two zMT isoforms, the result found in the

promoter study cannot reflect the zMT induction for all MT mRNA level. This may also be the other explanation for the low induction in the promoter level after the $Cu^{2+}$ exposure, if MT-II gene did not respond well to $Cu^{2+}$.

Table 4-1: Maximum fold inductions in MT mRNA, zMT-II gene promoter activities and MTF-1 mRNA caused by different heavy metal ions in SJD and ZFL cells

| | | $Zn^{2+}$ | $Cd^{2+}$ | $Cu^{2+}$ | $Hg^{+}$ | $As^{3+}$ | $As^{5+}$ | $Cr^{3+}$ | $Cr^{6+}$ |
|---|---|---|---|---|---|---|---|---|---|
| zMT mRNA | SJD | 113 ⊕ | 234 ⊕ | 21.0 ※ | 14.1 ⊕ | 153 * | 660 * | 3.00 ※ | 5.35 ⊕ |
| | ZFL | 125 ⊕ | 254 ※ | 80.8 ※ | 51.8 ※ | 4.93 ⊕ | - | - | - |
| zMT-1.4-luc | SJD | 13.1 ※ | 6.53 ⊕ | - | 3.16 * | 1.86 * | - | - | - |
| | ZFL | 6.78 ※ | 4.81 * | - | - | 1.74 * | - | - | - |
| MTF-1 mRNA | SJD | - | 10.9 ⊕ | - | - | 8.64 ⊕ | 5.41 ⊕ | 2.05 ⊕ | 3.37 ⊕ |
| | ZFL | - | 9.45 * | - | 5.41 ⊕ | - | - | - | - |

※ positive does response ; * negative does response;

⊕ bell shape response curve ; - : No significant induction was observed

## 4.5 Mechanism of MT gene expression and the MTF-1 mRNA inductions in SJD and ZFL cell lines .

Previous studies shown MT gene expression is induced by heavy metal ions through several mechanisms: facilitate the nuclear translocation of MTF-1, induce the DNA binding and phosphorylate MTF-1 protein (Smirnova *et al.*, 2000; Zhang *et al.*,

2001). Under normal condition MTF-1 in resting cells localizes to the cytoplasm, and it enters the nucleus under stress conditions. Zinc induction works by direct metal binding to the MTF-1 zinc fingers, but the exact signaling pathways for transcriptional activation remain to be established. Even though other metals, such as cadmium and copper, also activate transcription via MTF-1, activation must be indirect since these metals cannot replace zinc in zinc finger binding (Heuchel *et al.,* 1994; Dalton *et al.,* 1997; Bittel *et al.,* 1998; Chen *et al.,* 1999; Bittel *et al,* 2000; Zhang *et al.,* 2000).

Different species shown different response to the heavy metal ions in MTF-1 translocation, nuclear translocation of human and mouse MTF-1 has been reported in mammalian cell lines. The MTF-1 signals could be detected only in the nucleus at 1 h of treatment with 100 uM Zn. However in the zebrafish study, zinc and cadmium ions were found able to mediate the nuclear translocation (Moffat and Denizeau, 1997; Chen *et al,* 2007).

Another critical step for MT gene expression is the DNA-binding. The zinc finger motifs of MTF-1 fold correctly only in the presence of transition metal ions. Several transition metals, including $Cd^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Hg^{2+}$, $Mn^{2+}$, $Ni^{2+}$, and $Zn^{2+}$,

can bind to the zinc finger motifs (Berg, 1990; Chen *et al.,* 2007). Electrophoretic mobility shift assays (EMSAs) using whole cell and nuclear extracts from various higher eukaryotic cell lines, as well as recombinant MTF-1 synthesized in vitro in a coupled transcription - translation (TnT) system, demonstrated that MTF-1 binding to the MT-I MRE is highly sensitive to EDTA and requires low micromolar concentrations of zinc (Westin and Schaffner, 1988; Heuchel *et al.,* 1994; Otsukaet *et al,* 1994; Dalton *et al.,* 1997; Bittel *et al.,* 1998; Laity and Andrews, 2007).

It is hypothesized that $Zn^{2+}$ binds to the zinc fingers of MTF-1, causing a conformational change in the protein and subsequent binding to the MRE (Heuchel *et al., 1994*). Besides, it has been demonstrated that other transition metals cannot activate the MTF-1 DNA-binding activity in mouse and human even they can bind to the zinc finger motifs (Bittel *et al.,* 19981 Chen *et al.,* 2007). However, in fish MT genes, different reports are available that the DNA-binding activity of MTF-1 from rainbow trout cells can be induced by $Zn^{2+}$, whereas Japanese pufferfish MTF-1 binds with MRE sequence in response to $Zn^{2+}$ as well as $Cd^{2+}$ (Auf der Maur *et al.,* 1999; Dalton *et al.,* 2000). The result in zebrafish is even conflicting, it was firstly found that only $Zn^{2+}$ can induce the MRE-MTF-1 binding activity (Dalton *et al.,* 2000) but $Cd^{2+}$ was found to be involved in the increase of binding activity besides zinc (Chen

*et al.,* 2007).

Another way for the heavy metal ions to induce MT gene expression is through the post-translational modification of MTF-1 through phosphorylation. A model was proposed that the regulation of MT transcription, via the MTF-1/MRE interaction, is controlled by multiple signal transduction cascades that affect MTF-1 phosphorylation. This model is based on several observations. First, MTF-1 contains several evolutionarily conserved, potential phosphorylation sites: protein kinase C (PKC), casein kinase II, and tyrosine kinase. Second, exposure of cells to activators of signal transduction cascades causes an increase in the steady-state level of MT mRNAs. Similarly, the addition of signal transduction inhibitors attenuates or abolishes metal-inducible MT mRNA expression (Nebes *et al.,* 1988; Garrett *et al.,* 1992; Kelly *et al.,* 1997; Yu *et al.,* 1997; Laychoch *et al.,* 2000; Adams *et al.,* 2002). It has been shown that the level of MTF-1 phosphorylation is modified after the exposure to $Zn^{2+}$ or $Cd^{2+}$ *in vivo* (Adams and Freedman, 2000; LaRochelle *et al.,* 2001; Saydam *et al.,* 2002). This may help to explain how $Cd^{2+}$ activate MT gene expression without inducing MTF-1 DNA binding activity.

There is also one possible mechanism in inducing the MT gene expression other

than the mechanisms described above, which is the increase of MTF-1 content in the cell. As MTF-1 is an important element in the MT gene expression, it is interesting to know whether the heavy metal ions alter the MTF-1 level and so to induce the production of MT protein. However this mechanism has been rejected by the findings of some studies: a strong increase of MT mRNA level was observed in mouse after the treatment of zinc and cadmium ions, while transcription of MTF-1 itself is only marginally (Auf der Maur *et al.,* 2000; Lichtlen and Schaffner, 2001). The study about the expression of MT gene and MTF-1 gene has also been carried out in zebrafish and similar result was found. Through the *in situ* hybridization assay, zinc and cadmium ions were found to be potent inducers in MT gene, however the expression of MTF-1 was not induced by these two heavy metal ions (Chen *et al,* 2007).

Results from previous studies are conflict with the result found in this project. The result in this project indicated that the MTF-1 mRNA level in SJD and ZFL cells can be increased by some of the heavy metal ion exposures, including the cadmium ion. The different findings can be explained by the different species used in the experiment (for the study in mouse) and the different assays used in the studies. Auf de Maur and his colleagues performed northern blot assay to determine the MTF-1

mRNA level while the Chen's group determined it through *in situ* hybridization. In this project, real time PCR was performed to quantify the MTF-1 gene expression, which is a much sensitive method when compare with the previous methods. In other words, sensitivity of the experiment would be another reason account for the different results obtained.

It is not difficult to find that all the studies reviewed above used $Zn^{2+}$ and $Cd^{2+}$ as the inducers; however the role of heavy metal ions like $Cu^{2+}$, $Hg^{2+}$, $As^{3+}$, $As^{5+}$, $Cr^{3+}$and $Cr^{6+}$ in the MT gene expression are seldom studied. To have a more complete picture, this project also studied these heavy metal ions to see whether there are different MT gene expression mechanisms in different heavy metal ions. Not all the heavy metal ions were able to cause induction in the MTF-1 mRNA level. After comparing the MTF-1 inductions in the two cell lines caused by various heavy metal ions, it can be concluded that $Cd^{2+}$ is the greatest MTF-1 inducer among the eight heavy metal ions, while $Zn^{2+}$ and $Cu^{2+}$ were not participated in the mediation of MTF-1. Beside $Cd^{2+}$, $As^{3+}$, $As^{5+}$, $Cr^{3+}$and $Cr^{6+}$ were MTF-1 inducers in SJD cell line. But for ZFL cell line, only $Hg^{2+}$ and $Cr^{3+}$ were able to cause significant inductions besides cadmium ion. Due to the promoter region of MTF-1 gene in zebrafish has not been cloned yet and the positive result in MTF-1 gene expression is quite a new

finding, further investigation is needed in order to know how the heavy metal ions induce MTF-1 gene and so to know the reason of their different responses.

The findings about MTF-1 gene expression are tried to relate to the zMT gene expression and to see whether it is a possible mechanism for the MT gene expression. The data are compared and shown on Table 4.1. Through the comparison, it can be seen that all heavy metal ions that able to induce MTF-1 gene are shown induction in zMT gene in mRNA level. This result indicated that MTF-1 gene induction is a possible mechanism for the MT gene expression as all heavy metal ion exposure that can induce MTF-1 mRNA would follows the MT mRNA induction.

For some heavy metal ions, which can cause inductions in zMT gene but no significant induction was shown in MTF-1 gene ($Cu^{2+}$, $Hg^{2+}$ and $Zn^{2+}$ exposures in SJD cell line; $Cu^{2+}$, $Zn^{2+}$ and $As^{3+}$ exposures in ZFL cell line). For these cases, it is believed that the zMT-II gene inductions are carried out by other mechanisms but not the induction of MTF-1. Similar to $Zn^{2+}$, it has been proven that $Zn^{2+}$ induced MT gene expression through the mechanisms of nuclear translocation, MRE-MTF-1 binding and MTF-1 phosphorylation (LaRochelle *et al., 2001;* Chen *et al., 2007*).

However even both of the zMT-II and MTF-1 gene expression were induced, the relationship between them is not directly proportional, for example 10.9-fold and 5.41-fold MTF-1 induction were observed in $Cd^{2+}$ and $As^{5+}$ exposures in SJD cell line respectively. However, only 200-fold zMT induction was observed in the exposure to $Cd^{2+}$ but a much greater fold induction (600-fold) was observed in $As^{5+}$ exposures. These results indicated that MTF-1 gene induction should not be the sole mechanism for the MT gene expression and the mechanisms should exist together and contributed in the MT gene expression in different extent.

**4.6 Concluding Remarks**

From the findings in this project, it can be seen that different heavy metal ions have different responses in the SJD and ZFL cell lines. Like the $EC_{50}$ values of $As^{3+}$, $As^{5+}$, $Cr^{3+}$ and $Cr^{6+}$, it was determined by the cellular uptake in the two cell lines. When compare the MT mRNA inductions in the two cell lines, $Cd^{2+}$ and $Zn^{2+}$ were found as two potent inducers. Surprisingly, $As^{3+}$ and $As^{5+}$ were also found as potent inducers in the SJD cell line, where $As^{5+}$ was even the most potent inducer which shown 600- fold MT mRNA induction in the SJD cells. When compare between the responsiveness of the two cell lines, SJD was found more responsive in the MT gene expression that all heavy metal ions studied in this project gave a significant MT

induction in mRNA level. Therefore, it is a better model when applying MT mRNA level as the biomarker.

In the study of the MT promoter, which should be belonged to the MT-II gene of zebrafish, ten MREs were recognized in the 1.4kb long promoter region. Again, $Zn^{2+}$ and $Cd^{2+}$ were found as the potent inducers of promoter activity in the two cell lines after performing the transcient luciferase assays indicated these two heavy metal ions were directly related to the MT gene expression by stimulating the promoter region of MT gene and hence the transcription of it. Although significant inductions were found in the promoter level, the degree is much smaller than the MT induction in mRNA level. This is due to the real-time PCR assay was detecting all MT isoforms in mRNA level (MT-A and MT-II) while the transcient luciferase assay was studying the activity of MT-II promoter only, and so makes the two results incomparable. Besides, the higher sensitivity of real-time PCR assay may account for the result differences in the two assays.

MTF-1 induction was found as a possible way to cause MT gene expression; however this mechanism is not applied in all heavy metal ion treatments and all cell lines. The different responses of MTF-1 induction in the two cell lines indicated that

different MT gene expression mechanisms were applied on different organs. When compare the result between the heavy metal ion treatments, $Zn^{2+}$, which regarded as the potent MT inducer, was not able to induce the MTF-1 level. Therefore it is believed the MT gene expression caused by $Zn^{2+}$ was not through the MTF-1 induction but other mechanisms like stimulating the nuclear translocation, MTF-1 phosporylation and MRE-MTF-1 binding. But for $Cd^{2+}$, MTF-1 induction is obvious mechanism in the MT gene expression. As different metal would trigger the MT gene in different mechanisms, to have a thorough understanding about the mechanism of MT gene expression, the MRE-MTF-1 binding and nuclear translocation of MTF-1 should also be studied after the different metal treatments. This can provide a complete picture about how does the MT gene regulated by different heavy metal ions.

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